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PCT/NZ03/00184

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 20 August 2002 with an application for Letters Patent number 520897 made byGlycox Corporation Limited.

I further certify that pursuant to a claim under Section 24(1) of the PatentsAct 1953, a direction was given that the application proceed in the name of PROTEMIX CORPORATION LIMITED.

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Dated 10 September 2003.

Neville Harris

Neville Harris
Commissioner of Patents, Trade Marks and Designs

COMMISSIONER OF PATENTS, TRADEMARKS & DESIGNS
NEW ZEALAND

330 High Street, Lower Hutt and 17 Toop Street, Seaview
PO Box 30687, Lower Hutt, New Zealand or DX SX 11129, Wellington

520897

SUBSTITUTION OF APPLICANT UNDER SECTION 24

NEW ZEALAND

PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

"Dosage Forms and Related Therapies"

We, GLYCOX CORPORATION LIMITED, a company duly incorporated under the laws of New Zealand of Level 4, 41 Shortland Street, Auckland, New Zealand, do hereby declare this invention to be described in the following statement:

The present invention relates to (particularly diabetic) heart and coronary disease therapies and more particularly to methods of

(A) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences) and/or coronary artery structure damage selected from at least media damage (the muscle layer) and intima damage (the endothelial layer) (and its consequences), and/or

(B) improving in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") any one or more of systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imagining, and/or

(C) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") disorders or the heart muscle, macrovascular disease, microvascular disease and plaque rupture of athereomatous lesions of major blood vessels (and consequences thereof)

reliant upon, as active ingredient(s), trientine (See Martindale 33rd edition, 1025.3), salts of trientine and/or metabolites thereof.

The invention also consists in methods of reversing in a patient at least some of any damage arising from diabetic kidney disease, diabetic nephropathy and/or copper accumulation in the kidney, and/or reversing in a patient at least some of any damage to the renal arteries reliant upon the abovementioned active ingredient(s).

The patient may have elevated copper levels.

Reference to "copper levels capable of diminishment" may mean elevated copper levels but not necessarily so. The term "capable of diminishment" refers to copper values

readily scavenged by the trientine chelating moiety as opposed, we believe, to those copper values less readily scavenged (likely non interstitially located copper values).

Included in the present invention are dosage forms of the active ingredient(s) and relates to related uses (including uses in the preparation of pharmaceutical compositions of trientine, salts of trientine and/or metabolites thereof). Such dosage forms are indicated for at least both diabetic and elevated copper level patients.

By way of background the following can be stated in respect of diabetic heart disease in the human being,

1. Worldwide prevalence of diabetes is increasing. Number of cases of type 2 diabetes projected to increase from 135 million in 2000 to more than 300 million in 2025. Increase is related to ageing of the population, increasing obesity, and low socio-economic status. See, WHO. The World Health Report 1997.

2. Mortality from diabetes has increased over the last decade whereas mortality from cardiovascular disease, stroke, and malignant diseases has remained static or declined. See, US Centre for Health Studies.

3. Causes of premature mortality in type 2 diabetes comprise cardiovascular disease, 58%; cerebrovascular disease, 12%; nephropathy, 3%; diabetic coma, 1%; malignancy, 11%; and infections 4%. See, Pickup J, Williams G eds. Handbook of diabetes, 2nd edition, 1999; p 24.

4. Diabetic heart disease is characterised by more severe coronary artery disease at a younger age, a 4-fold increased frequency of heart failure post-acute myocardial infarction and a disproportionate increase in left ventricular hypertrophy. See Struthers AD, Morris Ad, Lancet 2002;359:1430-2.

5. Patients with type 2 diabetes manifest a disproportionate increase in mortality within the first 24-hours post-acute myocardial infarction. Acute intervention can ameliorate this risk. See, Malmberg K Br Med J 1997;314:1512-5.

Much of the above is equally applicable to diabetic coronary artery structure.

PCT/NZ99/00161 (published as WO00/18392 on 6 April 2000) has disclosed a method of treating a mammalian patient predisposed to and/or suffering from diabetes mellitus with a view to minimising the consequences of macrovascular and microvascular

damage to the patent which comprises, in addition to any treatment in order to control blood glucose levels, at least periodically inhibiting or antagonizing fructosamine oxidase enzyme activity in the patient. An assay for such activity is disclosed in their PCT/NZ99/00160 (published as WO00/18891 on 6 April 2000).

A range of different agents capable of acting as fructosamine oxidase inhibitors and/or antagonists were disclosed in PCT/NZ99/00161. These included copper chelating agents, substrate analogues and hydrazine compounds.

The full contents of the aforementioned specifications are here included by way of reference.

We have hypothesised that reduction in available free copper does have an affect in preventing macrovascular, microvascular and/or toxic/metabolic diseases of the kind hereinafter exemplified and in tissue repair processes. This is irrespective of the glucose metabolism of the patient.

We have also hypothesized that cardiovascular accumulation of redox-active transition metal ions is responsible for many of the adverse outcomes in diabetes. Under physiological conditions, injury to a target organ is sensed by distant stem cells, which migrate to the site of damage then undergo alternate stem cell differentiation; these events promote structural and functional repair. However, the accumulation of redox-active transition metals, particularly copper in cardiac or vascular tissues in subjects with diabetes is accompanied by a suppression of the normal tissue regeneration effected by the migration of stem cells. Elevated tissue levels of copper suppress these normal biological behaviours of such undifferentiated cells. Conditions occurring in the context of diabetes or impaired glucose tolerance, in which the suppression of normal stem cell responses can cause impairment of normal tissue responses, include the following:

1. Cardiac failure
2. Acute myocardial infarction
3. Wound healing and ulceration
4. Tissue damage caused by infection
5. Diabetic kidney damage

Conditions in which therapy to lower copper values in diabetic patients (ie; with IGT or Type 2 Diabetes Mellitus) is liable to prove beneficial include at least the following:

1. HEART FAILURE IN THE CONTEXT OF DIABETES

Significant regeneration of cardiac tissues can occur within a few days of cardiac transplantation. The likely mechanism is migration of stem cells from extra-cardiac sites to the heart, with subsequent differentiation of such cells into various specialized cardiac cells, including myocardial, endothelial and coronary vascular cells. We believe that copper accumulation in cardiac tissues is likely to severely impair these regenerative responses. Hence a role for acute intravenous therapy with a copper chelator in the treatment of diabetic heart failure.

2. MYOCARDIAL INFARCTION IN THE CONTEXT OF DIABETES.

Myocardial infarction is accompanied by proliferation of cells in the ventricular myocardium when MI occurs in the context of diabetes, the presence of elevated tissue levels of redox-active transition metals suppresses normal stem cell responses, resulting in impaired structural and functional repair of damaged tissues. Up to 20% of cells in the heart may be replaced by stem cell migration from extra-ventricular sites, as soon as four days after cardiac transplantation. These observations suggest that treatment of AMI in the context of diabetes will be improved by acute (if necessary, parenteral) as well as by subsequent chronic administration of chelators. The mechanism of the impairment of cardiac function in diabetes is likely a toxic effect of accumulated transition metals on tissue dynamics, resulting in impaired tissue regeneration caused in turn by suppression of normal stem cell responses, which mediate physiological tissue regeneration by migration to damaged tissue from external sites.

3. WOUND HEALING AND ULCERATION IN THE CONTEXT OF DIABETES

The processes of normal tissue repair require intervention of mobilizing stem cells, which effect repair of the various layers of blood vessels, for example. We believe that an accumulation of transition metals (particularly copper) in vascular tissues causes the

impaired tissue behaviour characteristic of diabetes, including impaired wound repair following surgery or trauma, and the exaggerated tendency to ulceration and poor healing of established ulcers. We believe that the treatment of diabetics with copper chelators before they undergo surgery, or in the context of traumatic tissue damage, is likely to be of benefit. It is probable that surgery in diabetics would have a better outcome if excess transition metals were removed from blood vessels prior to surgery. This may need to be accomplished on either an acute basis (with parenteral therapy) or on a more chronic basis (with oral therapy) prior to actual surgery.

4. SOFT TISSUE DAMAGE RESULTING FROM INFECTION AND OCCURING IN THE CONTEXT OF DIABETES OR IMPAIRED GLUCOSE TOLERANCE

We believe the processes of normal tissue repair following infection require intervention of mobilized stem cells, which migrate to sites of tissue damage to effect tissue regeneration and repair, for example, of the various layers of blood vessels. Such tissue damage will be impaired by suppressed stem cell responses, such as those caused by the build up of redox-active transition metals (particularly copper) in tissues, for examples the walls of blood vessels.

5. KIDNEY DAMAGE OCCURING IN THE CONTEXT OF DIABETES

We believe that impaired stem cell responses in the kidneys of diabetics contribute to diabetic nephropathy and renal failure. We believe that treatment of diabetics having kidney failure by administration of a copper chelator will improve organ regeneration by restoring normal tissue healing by allowing stem cells to migrate and differentiate normally.

However, even in the non diabetic mammal and even in a mammal without a glucose mechanism abnormality, we have hypothesized a reduction in extra-cellular copper values is advantageous in that such lower levels will lead to one or both a reduction in copper mediated tissue damage and improved tissue repair by restoration of normal tissue stem cell responses.

In our own studies (using the streptozocin-diabetic (STZ) rat model) we have found a high frequency of tissue damage in the heart tissue and coronary artery tissue in severely diabetic animals. This reflects what is found in man.

We now more firmly take the view that copper values (and particularly copper II) not bound internally of cells is available to mediate (together with available reducing substances) the generation of damaging free radicals that have a role in both tissue damage and impairment of stem cell mediated repair of such tissue. This is irrespective of diabetic status but we believe is more prevalent in diabetic rats and other mammals including human beings.

In respect of such damage and repair impairment we propose a diminishment in available free copper values as being an appropriate preventive and/or treatment approach for diabetic patients or any patient (particularly a patient not suffering Wilson Disease) who has elevated copper levels.

Our agent of choice is trientine, preferably as an acid addition salt.

Alternative names for trientine include *N,N'-Bis(2-aminoethyl)-1,2-ethanedi-amine; triethylenetetramine;* 1,8-diamino-3,6-diazaoctane; 3,6-diazaoctane-1,8-diamine; 1,4,7,10-tetraazadecane; trien; TETA; TECZA and triene.

Reference made herein to "trientine" refers to the moiety substantially of the structure depicted but can include analogues thereof which are prodrugs of the active copper chelating moiety or metabolite of trientine. Salts of trientine (which optionally can be salts of a prodrug of the trientine copper chelating moiety or metabolite) are preferably acid addition salts such as, for example, those of suitable mineral or organic acids, eg; the hydrochlorides, maleates, citrates, tartrates, etc.

Salts of trientine (such as acid addition salts, eg; trientine dihydrochloride) act as copper-chelating agents, which aids the elimination of copper from the body by forming a stable soluble complex that is readily excreted by the kidney.

Trientine, a strongly basic moiety, with its multiple nitrogens can be converted into a large number of suitable associated acid addition salts using an acid, for example, by reaction of stoichiometrically equivalent amounts of trietine and of the acid in an inert solvent such as ethanol or water and subsequent evaporation if the dosage form is best

formulated from a dry salt. Possible acids for this reaction are in particular those which yield physiologically acceptable salts. Thus inorganic acids can be used, e.g. sulfuric acid, nitric acid, hydrohalic acids such as hydrochloric acid or hydrobromic acid, phosphoric acids such as orthophosphoric acid, sulfamic acid. Furthermore organic acids, can be used, in particular aliphatic, alicyclic, araliphatic, aromatic or heterocyclic mono- or polybasic carboxylic, sulfonic or sulfuric acids, (e.g. formic acid, acetic acid, propionic acid, pivalic acid, diethylacetic acid, malonic acid, succinic acid, pimelic acid, fumaric acid, maleic acid, lactic acid, tartaric acid, malic acid, citric acid, gluconic acid, ascorbic acid, nicotinic acid, isonicotinic acid, methane-or ethanesulfonic acid, ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, naphthalenemono-and-disulfonic acids and laurylsulfuric acid).

The trientine moieties can also be in the form of quarternary ammonium salts in which the nitrogen atom carries a suitable organic group such as an alkyl, alkenyl, alkynyl or aralkyl moiety.

Preferably the trientine moieties are in the form of a compound or buffered in solution and/or suspension to a near neutral pH much lower than the pH of 14 of trientine itself.

Suitable anions may include citrate, isocitrate, α-Ketoglutarate, Succinate, Fumarate, Malate, Oxaloacetate, Acetate and pyruvate.

Trientine moieties (preferably delivered as a salts of trientine (such as acid addition salts, eg; trientine dihydrochloride) act as copper-chelating agents, which aids the elimination of copper from the body by forming a stable soluble complex that is readily excreted by the kidney.

The presumed site of action of the chelating trientine moiety of a salt such as trientine dihydrochloride is the removal of loosely bound copper from the body and in particular from the cardiac extracellular matrix and the coronary extracellular matrix.

Bioavailabilities of the active species of trientine dihydrochloride after oral administration is low (<10%) due to poor absorption and marked first-pass metabolism. Trientine dihydrochloride and its transformed metabolite, *N*-acetyl-trientine hydrochloride, are both capable of binding copper, although the chelating activity of the

analogue *N*-acetyl-trientine hydrochloride is significantly lower than trientine dihydrochloride. See, Kodama H. *Life Sciences* 1997;61:899-907.

Absorption of trientine dihydrochloride is adversely affected by food, mineral supplements and other drugs.

We have now shown in the STZ rat model for both diabetic and non diabetic man a reduction in available free copper does have an affect in reversing in the diabetic rat both (i) cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extra cellular space and increased deposition of extra cellular matrix (and its consequences) and (ii) coronary artery structure damage (and its consequences). We have also shown amelioration. In so showing reversal of damage in the STZ, we have found a dose relativity for man insofar as the copper scavenging into the urine is concerned.

Under physiological conditions we believe injury to the cardiac structure is sensed by distant stem cells, which migrate to the site of damage then undergo alternate stem cell differentiation; these events promote structural and functional repair. However, the accumulation of redox-active transition metals, particularly copper in cardiac tissues and coronary arteries in subjects with diabetes we believe is accompanied by a suppression of the normal tissue regeneration effected by the migration of stem cells. Elevated tissue levels of copper suppress these normal biological behaviours of such undifferentiated cells.

Even in the non diabetic mammal (e.g. without Type 2 Diabetes mellitus) and even in a mammal without a glucose mechanism abnormality (e.g. without IGT), we believe a reduction in extra-cellular copper values is advantageous in that such lower levels will lead to one or both a reduction in copper mediated tissue damage and improved tissue repair by restoration of normal tissue stem cell responses.

It is an object of the present invention to provide sustained, controlled and/or extended release dosage forms useful for taking advantage of this prospect for the purpose of amelioration of such structure damage and damage reversal all irrespective of whether or not our hypothesis or proposals as to mode of action are correct.

Such damage includes cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences) and/or coronary artery structure damage selected from at least media damage (the muscle layer) and intima damage (the endothelial layer) (and its consequences), whether or not the hypotheses or proposals as to mode of action are correct.

It is another object to provide uses and dosage forms applicable instead or as well to improve in a human being or other mammal (preferably diabetic and/or with a raised copper level) ("the patient") any one or more of systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imaging), whether or not the hypotheses or proposals as to mode of action are correct.

It is another object of the present invention to provide methods of treatment and related methods, uses and pharmaceutical compositions that ameliorate, prevent or treat any one or more disease states of the cardiovascular tree (including the heart) and dependent organs (eg; retina, kidney, nerves, etc.) exacerbated by elevated non-intracellar free copper values levels, whether or not the hypotheses or proposals as to mode of action are correct.

Reference herein to diseases of the cardiovascular tree and diseases of dependent organs includes any one or more of

(i) **disorders of the heart muscle** (cardiomyopathy or myocarditis) such as idiopathic cardiomyopathy, metabolic cardiomyopathy which includes diabetic cardiomyopathy, alcoholic cardiomyopathy, drug-induced cardiomyopathy, ischemic cardiomyopathy, and hypertensive cardiomyopathy,or

(ii) atheromatous disorders of the major blood vessels (**macrovascular disease**) such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the femoral arteries, and the popliteal arteries,or

(iii) toxic, drug-induced, and metabolic (including hypertensive and/or diabetic disorders of small blood vessels (**microvascular disease**) such as the retinal arterioles, the glomerular arterioles, the vasa nervorum, cardiac arterioles, and associated capillary beds of the eye, the kidney, the heart, and the central and peripheral nervous systems,or

(iv) **plaque rupture of atheromatous lesions of major blood vessels** such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the fermoral arteries and the popliteal arteries.

The present invention relates to any such ailments and their treatment irrespective (unless otherwise stated) of any diabetic and/or glucose abnormality state of the mammalian patient.

Compliance of a dosage regime is always essential in order to derive the best benefit from a treatment regime. The present invention recognises a benefit from sustained release dosage forms that can provide such levels of sustained delivery to a patient as are required to elicit the advantages now seen from the prospect of lower overall dose delivery of trientine formulations when one compares them to the twice a day multiple dosage oral regimes hitherto used with trientine formulations for Wilson's disease.

The present invention in one aspect consists in **a parenteral formulation or dosage form** capable of delivery of an effective amount of trientine hydrochloride and/or its metabolites when administered or self administered to a human being or other mammal (preferably diabetic or predisposed thereto or with elevated copper levels) sufficient

(A) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences), and/or coronary artery structure damage (and its consequences),

(B) improving in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient")

any one or more systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imaging),

(C) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") disorders of the heart muscle, macrovascular disease, microvascular disease and plaque rupture of athereomatous lesions of major blood vessels (and the consequences thereof), and/or

(D) reversal of at least some of any damage arising from diabetic kidney disease, diabetic nephropathy and/or copper accumulation in the kidney and/or reversal of at least some of any damage to the renal arteries.

In certain circumstances, oral administration of trientine is not possible or desirable. For example, acute myocardial infarction is often accompanied by nausea and vomiting, rendering the oral route of administration ineffective. Gastric emptying may also be delayed under these conditions. There is thus a need for a parenteral (eg; an injectable) composition containing trientine or a pharmaceutically acceptable salt thereof at least for the treatment of patients with acute coronary syndrome.

Suitable parenteral forms include solutions, suspensions, emulsions etc. that can be administered parenterally by either subcutaneous injections, intravenous, intramuscular, intradermal, intrastemal injection or infusion techniques.

Accordingly in another aspect the present invention in one aspect consists in a **method** of

(A) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences), and/or coronary artery structure damage (and its consequences),

(B) improving in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient")

any one or more systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imaging),

(C) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") disorders of the heart muscle, macrovascular disease, microvascular disease and plaque rupture of athereomatous lesions of major blood vessels (and the consequences thereof), and/or

(D) reversal of at least some of any damage arising from diabetic kidney disease, diabetic nephropathy and/or copper accumulation in the kidney and/or reversal of at least some of any damage to the renal arteries.

which method comprises or includes the step of administration and/or self administration to the patient an effective amount of a parenteral formulation or dosage form, said formulation or dosage form having as the or an active agent a suitable trientine moiety (e.g. trientine, at least one salt of trientine and/or at least one metabolite of trientine and/or its salt(s) ("trientine" including analogues thereof and/or prodrugs thereof)).

Preferably the effective amount is sufficient to provide effective chelation of copper for an overall diminishment thereof in the patient.

Preferably the effective amount is of trientine dihydrochloride.

In a further aspect the present invention consists in **the use** of a suitable trientine moiety (e.g. trientine, at least one salt of trientine and/or at least one metabolite of trientine and/or its salt(s) (the "active agent(s)")), together with other material(s) appropriate for a parenteral formulation or dosage form, **in the manufacture of a parenteral formulation or dosage form** useful for

(A) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences), and/or coronary artery structure damage (and its consequences),

(B) improving in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") any one or more systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imaging),

(C) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") disorders of the heart muscle, macrovascular disease, microvascular disease and plaque rupture of athereomatous lesions of major blood vessels (and the consequences thereof), and/or

(D) reversal of at least some of any damage arising from diabetic kidney disease, diabetic nephropathy and/or copper accumulation in the kidney and/or reversal of at least some of any damage to the renal arteries.

We have determined in our trials referred to hereinafter that a divided dose of 1.2 g/day is effective for and yet (insofar as an instantaneous body level is concerned) in excess of dosage levels to be required chronically in practice for the purpose of amelioration and/or reversal of cardiac structure damage and/or coronary artery structure damage. Such a dose rate of 1.2 g/day is capable of being provided parenterally.

In another aspect the present invention consists in a method of administering an effective amount of Trientine formulated in a parenterally acceptable formulation.

Preferably said formulation is suitable for use in the treatment of any of heart failure, diabetic heart disease, acute coronary syndrome, hypertensive heart disease, ischaemic heart disease, coronary artery disease, peripheral arterial disease, Wilson's disease, or any form of cancer.

Preferably said formulation contains an effective dosage unit to the patient of the trientine from 1 mg to 600mg per uniit.

Preferably the total daily dose rate is from between 5gms to 1mg.

Preferably the dosage unit will maintain a constant blood plasma concentration from between 1 hour to 24 hour.

In another aspect of the present invention consists in a formulation of trientine that maintains constant plasma concentrations of the drug for extended periods and is effective in removing copper from the body of patients with any of heart failure, diabetic heart disease, acute coronary syndrome, hypertensive heart disease, ischaemic heart disease, coronary artery disease, peripheral arterial disease, Wilson's disease, or any form of cancer.

Reference herein to "elevated" in relation to the presence of copper values will include humans having at least 10 mcg free copper/dL of serum when measured as discussed by Merck & Co Inc below.

A measurement of free copper [which equals total plasma copper minus ceruloplasmin-bound copper] can be made using the procedure disclosed in the Merck & Co Inc datasheet (www.Merck.com) for SYPRINE® (trientine dihydrochloride) capsules where they state in respect of the use of trientine dihydrochloride for the copper values excesses of Wilson's Disease:

"The most reliable index for monitoring treatment is the determination of free copper in the serum, which equals the difference between quantitatively determined total copper and ceruloplasmin-copper. Adequately treated patients will usually have less than 10 mcg free copper/dL of serum.

Therapy may be monitored with a 24 hour urinary copper analysis periodically (i.e. every 6-12 months). Urine must be collected in copper-free glassware. Since a low copper diet should keep copper absorption down to less than one milligram a day, the patient probably will be in the desired state of negative copper balance if 0.5 to 1.0 milligram of copper is present in a 24-hour collection of urine"

We have conducted studies reliant on trientine dihydrochloride in the STZ rat model as well in humans and wish to describe the invention further by reference to the accompanying drawings in which:

Figure 1 (**Figure 1A** in colour and **Figure 1B** (the same as Figure 1A) in black and white) shows for the STZ rat model, animals made diabetic for more than six weeks, having damaged cardiac structure with atrophy and loss of myocytes, expansion of the extracellular space, and increased deposition of extracellular matrix; these differences are

observed between showing the non diabetic STZ rat cardiac tissue, the untreated diabetic rat tissue and the trientine dihydrochloride treated diabetic rat tissue,

Figure 2A shows in STZ diabetic rats compared with non diabetic rats cardiac function impairment that is largely corrected by chronic oral therapy with trientine dihydrochloride,

Figure 2B shows functional survival of working hearts arising from the isolated working heart model to form the basis of the data generation for Figure 2A,

Figure 3 shows how doses of trientine hydrochloride modifies copper excretion in the urine,

Figure 4 shows the absolute weight change with time of the period of the experiment,

Figure 5 is a diagram showing the bodyweight of the animals changing over the time period of the experiment,

Figure 6 shows the glucose levels of the animals changing over the time period of the experiment,

Figure 7 is a diagram showing cardiac output at various filling pressures,

Figure 8 is a diagram showing the coronary flow at various filling pressures,

Figure 8A is a diagram showing the coronary flow of Figure 8 normalised to final cardiac weight,

Figure 9 is a diagram showing the aortic flow with increasing preload,

Figure 10 is a diagram showing the peak pressure developed in the left ventricle at various filling pressures,

Figure 11 is a diagram showing the maximal rate of positive change in pressure development in the ventricle in response to increasing preload,

Figure 12 is a diagram showing the corresponding maximal rate of decrease in ventricular pressure in response to increasing preload,

Figure 13 shows the maximal rate of increase in pressure in the aortic outflow,

Figure 14 shows the corresponding maximal rate of decrease in the aortic pressure in response to increasing preload,

Figure 15 shows the percentage of functionally surviving hearts at each afterload pressure,

Figure 16 shows the cardiac output in response to increasing afterload,

Figure 17A shows the absolute change in coronary flow in response to increasing afterload,

Figure 17B shows the increases in coronary flow in response to increasing afterload normalized to heart weight,

Figure 18 shows the peak pressure developed in the left ventricle in response to increasing afterload,

Figure 19 shows the maximum rate of positive change in pressure development in the ventricle in response to increasing afterload,

Figure 20 shows the corresponding maximum rate of decrease in ventricular pressure in response to increasing afterload,

Figure 21 shows the maximum rate of positive change in pressure within the aortic outflow in response to increasing afterload,

Figure 22 shows the corresponding maximal rate of decrease in aortic pressure in response to increasing afterload,

Figure 23A shows diagrammatically how the extracted heart was attached to the modified apparatus,

Figure 23B shows diagrammatically in more detail the extracted heart as it was attached to the modified apparatus,

Figure 24 shows the urine excretion in diabetic and non diabetic animals in response to increasing doses of trientine,

Figure 25 shows volume of or an equivalent volume of saline (control) urine excreted in non diabetic and diabetic animals receiving increasing doses of trientine or an equivalent volume of saline,

Figure 26 shows copper excretion in the urine of diabetic and non diabetic animals receiving increasing doses of trientine or an equivalent volume of saline,

Figure 27 shows the same information in Figure 26 as urinary copper excretion per gram of bodyweight,

Figure 28 shows the total amount of copper excreted in non diabetic and diabetic animals administered saline or trientine,

Figure 29 shows the total amount of copper excreted per gram of bodyweight in animals receiving trientine or saline,

Figure 30 shows the iron excretion in urine of diabetic and non diabetic animals receiving increasing doses of trientine or an equivalent volume of saline,

Figure 31 shows the urinary iron excretion per gram of bodyweight in diabetic and non diabetic animals receiving trientine or saline.,

Figure 32 shows the total urinary iron excretion in non diabetic and diabetic animals administered saline,

Figure 33 shows the total urinary iron excretion per gram of bodyweight in animals receiving trientine or saline,

Figure 34 shows the percentage of surviving hearts at each afterload pressure,

Figure 35 is a table comparing the copper and iron excretion in the animals receiving trientine or saline, which is a statistical analysis using a mixed linear model,

Figure 36 is a plasma concentration-time profiles of trientine after oral administration to four male patients, and

Figure 37 is a plasma concentration-time profiles of trientine after oral administration to four female patients..

In the STZ rat model, animals made diabetic for more than 6 weeks show damaged cardiac structure with atrophy and loss of myocytes, expansion of the extracellular space, and increased deposition of extracellular matrix. These appearances are both ameliorated and reversed by chronic oral therapy with trientine dihydrochloride. See, Figures 1A & 1B.

Using the isolated working heart model, cardiac function is severely impaired in STZ diabetic rats compared with non-diabetic animals. Cardiac dysfunction is largely corrected by chronic oral therapy with trientine hydrochloride. See, Figures 2A & 2B. This is more clearly set out in the experimentation studies below.

EXPERIMENTS CONDUCTED

INTRODUCTION

Increased tissue copper has been implicated in mechanisms leading to diabetic nerve damage. We therefore hypothesized that tissue accumulation of trace metals might play a role in the mechanisms of diabetic damage in other tissues. Histological evidence from our earlier studies showed that 6 months of treatment with trientine appears to protect the hearts of diabetic Wistar rats from development of diabetic damage (cardiomyopathy) as judged by histology. In this study we investigated the doses of trientine required for copper and iron to be excreted in the urine, and also any possible difference between the excretion of these metals in diabetic and nondiabetic animals.

AIM

1. To compare the excretion profiles of copper and iron in the urine of normal and diabetic rats after acute intravenous administration of varying doses of trientine.
2. To ascertain that the acute intravenous administration of trientine has no acute adverse cardiovascular side effects.

METHODS

All methods used in this study were approved by the University of Auckland Animal Ethics Committee and were in accordance with The Animals Protection Act and Regulations of New Zealand.

Induction of diabetes.

Male Wistar rats (n = 28, 303 ± 2.9 g) were divided randomly into diabetic and nondiabetic groups. Following induction of anesthesia (5% halothane and 2l.min^{-1} O_2), animals in the diabetic group received a single intravenous dose of streptozotocin (STZ, 55mg.kg^{-1} body weight, Sigma; St. Louis, MO) in 0.5 ml saline administered via the tail vein. Nondiabetic animals received an equivalent volume of saline. Following injection, both diabetic and nondiabetic rats were housed in like-pairs and provided with access to normal rat chow (Diet 86 pellets; New Zealand Stock Feeds, Auckland, NZ) and

deionized water *ad libitum*. Blood glucose and body weight were measure at day 3 following STZ/saline injection and then weekly throughout the study. Diabetes was identified by polydipsia, polyuria and hyperglycemia (> 11 $mmol.l^{-1}$, Advantage II, Roche Diagnostics, NZ Ltd).

Experimental procedure.

Six to seven weeks (mean = 44 ± 1 days) after administration of STZ, animals underwent either a control or drug experimental protocol. All animals were fasted overnight prior to surgery but continued to have *ad libitum* access to deionized water. Induction and maintenance of surgical anesthesia was by 3 - 5% halothane and 2l. min^{-1} O_2. The femoral artery and vein were cannulated with a solid-state blood pressure transducer (Mikrotip™ 1.4F, Millar Instruments, Texas, USA) and a saline filled PE 50 catheter respectively. The ureters were exposed via a midline abdominal incision, cannulated using polyethylene catheters (external diameter 0.9 mm, internal diameter 0.5 mm) and the wound sutured closed. The trachea was cannulated and the animal ventilated at 70-80 $breaths.min^{-1}$ with air supplemented with O_2 (Pressure Controlled Ventilator, Kent Scientific, Connecticut, USA). The respiratory rate and end-tidal pressure (10-15 cmH_2O) were adjusted to maintain end-tidal CO_2 at 35-40 mmHg (SC-300 CO_2 Monitor, Pryon Corporation, Wisconsin, USA). Body temperature was maintained at 37°C throughout surgery and the experiment by a heating pad. Estimated fluid loss was replaced with intravenous administration of 154 $mmol.l^{-1}$ NaCl solution at a rate of 5 $ml.kg^{-1}.h^{-1}$.

Following surgery and a 20 min stabilization period, the experimental protocol was started. Trientine was administered intravenously over 60 s in hourly doses of increasing strength (0.1, 1.0, 10 and 100 $mg.kg^{-1}$ in 75 µl saline followed by 125 µl saline flush). Control animals received an equivalent volume of saline. Urine was collected in 15 min aliquots throughout the experiment in pre-weighed polyethylene eppendorf tubes. At the end of the experiment a terminal blood sample was taken by cardiac puncture and the separated serum stored at -80°C until future analysis. Hearts were removed through a rapid mid-sternal thoracotomy and processed as described below.

Data acquisition.

Mean arterial pressure (MAP), heart rate (HR, derived from the MAP waveform) oxygen saturation (Nonin 8600V Pulse Oximeter, Nonin Medical Inc., Minnesota, USA) and core body temperature, were all continuously monitored throughout the experiment using a PowerLab/16s
data acquisition module (AD Instruments, Australia). Calibrated signals were displayed on screen and saved to disc as 2 s averages of each variable.

Urine and tissue analysis.

Instrumentation: A Perkin Elmer (PE) Model 3100 Atomic Absorption Spectrophotometer equipped with a PE HGA-600 Graphite Furnace and PE AS-60 Furnace Autosampler was used for Cu and Fe determinations in urine. Deuterium background correction was employed. A Cu or Fe hollow-cathode lamp (Perkin Elmer Corporation) was used and operated at either 10 W (Cu) or 15 W (Fe). The 324.8 nm atomic line was used for Cu and the 248.3 nm atomic line for Fe. The slit width for both Cu and Fe was 0.7 nm. Pyrolytically coated graphite tubes were used for all analyses. The injection volume was 20 µL. A typical graphite furnace temperature program is shown below (table 1).

Table 1: GF-AAS temperature program

Procedure	*Temp / ℃*	*Ramp / s*	*Hold / s*	*Int. Flow / mL min^{-1}*
Drying	90	1	5	300
	120	60	5	300
Pre-treatment	1250*	20	10	300
	20	1	10	300
Atomization – Cu / Fe	2300 / 2500	1	8	0
Post-treatment	2600	1	5	300

- A pre-treatment temperature of 1050 °C was used for tissue digest analyses

Cu, Fe and Zn in tissue digests were also determined at Hill Laboratories (Hamilton, New Zealand) using either a PE Sciex Elan-6000 or PE Sciex Elan-6100 DRC ICP-MS. The operating parameters are summarised in the table below (table 2).

Table 2: Instrumental operating parameters for ICP-MS

Parameter	Value
Inductively coupled plasma	
Radiofrequency power	1500 W
Argon plasma gas flow rate	15 $l.min^{-1}$
Argon auxiliary gas flow rate	1.2 $l.min^{-1}$
Argon nebuliser gas flow rate	0.89 $l.min^{-1}$
Interface	
Sampler cone and orifice diameter	Ni / 1.1 mm
Skimmer cone and orifice diameter	Ni / 0.9 mm
Data acquisition parameters	
Scanning mode	Peak hopping
Dwell time	30 ms (Cu, Zn) / 100 ms (Fe)
Sweeps / replicate	20
Replicates	3
Sample uptake rate	1 $ml.min^{-1}$

Reagents: All reagents used were of the highest purity available and at least of analytical grade. GF-AAS standard working solutions of Cu and Fe were prepared by stepwise dilution of 1000 $mg.l^{-1}$ (Spectrosol standard solutions; BDH). Water was purified by a Millipore Milli-Q ultra-pure water system to a resistivity of 18 MΩ. Standard Reference Material 1577b Bovine Liver was obtained from the National Institute of Standards and Technology and used to evaluate the efficiency of tissue digestion. The results obtained are reported below (table 3).

Table 3: GF-AAS and ICP-MS results for NIST SRM 1577b bovine liver*

Element	*Certified value*	*GF-AAS*	*ICP-MS*
Cu	160 ± 8	142 ± 12	164 ± 12
Fe	184 ± 15	182 ± 21	166 ± 14
Zn	127 ± 16	–	155 ± 42

* Measured in $\mu g.g^{-1}$ of dry matter.

Sample pretreatment:

Urine: Urine was collected in pre-weighed 1.5 ml micro test tubes (eppendorf). After reweighing, the urine specimens were centrifuged and the supernatant diluted 25:1 with 0.02 M 69 % Aristar grade HNO_3. The sample was stored at 4 °C prior to GF-AAS analysis. If it was necessary to store a sample for a period in excess of 2 weeks, it was frozen and kept at –20 °C.

Heart: Following removal from the animal, the heart was cleaned of excess tissue, rinsed in buffer to remove excess blood, blotted dry and a wet ventricular weight recorded. Using titanium instruments a segment of left ventricular muscle was dissected and placed in a pre-weighed 5.0 ml polystyrene tube. The sample was freeze-dried overnight to constant weight before 0.45 ml of 69% Aristar grade HNO_3 was added. The sample tube was heated in a water bath at 65 °C for 60 minutes. The sample was brought to 4.5 ml with Milli-Q H_2O. The resulting solution was diluted 2:1 in order to reduce the HNO_3 concentration below the maximum permitted for ICP-MS analysis. The remaining left ventricular tissue was stored in 10% formalin and later processed for transmission electron microscopic examination and histochemical analysis.

Serum: Terminal blood samples were centrifuged and serum treated and stored as per urine until analysis. From the trace metal content of serum from the terminal blood

sample and urine collected over the final hour of the experiment, renal clearance was calculated using the following equation:

$$\text{renal clearance of trace metal} = \frac{\text{concentration of metal in urine } (\mu g.\ \mu l^{-1}) * \text{rate of urine flow } (\mu l.min^{-1})}{\text{concentration of metal in serum } (\mu g.\ \mu l^{-1})}$$

Statistical analysis.

All values are expressed as mean ± SEM and P values < 0.05 were considered statistically significant. Student's unpaired t-test was initially used to test for weight and glucose differences between the diabetic and control groups. For comparison of responses during drug exposure, statistical analyses were performed using analysis of variance (Statistica for Windows v.6.1, SAS Institute Inc., Calfornia, USA). Subsequent statistical analysis was performed using a mixed model repeated measures ANOVA design (see: Figure 35 and Table 7 *below*).

RESULTS

Table 4.: Blood glucose, body weight and food consumption in diabetic versus nondiabetic animals.

	Diabetic	**Nondiabetic**
Body weight prior to STZ/saline	303 ± 3 g	303 ± 3 g
Blood glucose 3 days following STZ/saline	*25 ± 2 mmol.l^{-1}	5 ± 0.2 mmol.l^{-1}
Daily food consumption	*58 ± 1 g	28 ± 1 g
Blood glucose on experimental day	*24 ± 1 mmol.l^{-1}	5 ± 0.2 mmol.l^{-1}
Body weight on experimental day	*264 ± 7 g	434 ± 9 g

Diabetic animals n = 14, nondiabetic animals n = 14. Values shown as mean ± SEM. Asterisk indicates a significant difference (P < 0.05).

Effects of STZ on blood glucose and body weight (Table 4)

Blood glucose increased to 25 ± 2 mmol.l^{-1} three days following STZ injection. Despite a greater daily food intake, diabetic animals lost weight whilst nondiabetic animals continued to gain weight during the 44 days following STZ/saline injection. On

the day of the experiment blood glucose levels were 24 ± 1 and 5 ± 0 mmol.l^{-1} and body weight 264 ± 7 g and 434 ± 9 g for diabetic and nondiabetic animals respectively.

Cardiovascular variables during infusion

Baseline levels of MAP during the control period prior to infusion were not significantly different between nondiabetic and diabetic animals (99 ± 4 mmHg). HR was significantly lower in diabetic than nondiabetic animals (287 ± 11 and 364 ± 9 bpm respectively, $P < 0.001$). Infusion of trientine or saline had no effect on these variables except at the highest dose where MAP decreased by a maximum of 19 ± 4 mmHg for the 2 min following administration and returned to pre-dose levels within 10 min. Body temperature and oxygen saturation remained stable in all animals throughout the experiment.

Urine excretion

Diabetic animals consistently excreted significantly more urine than nondiabetic animals except in response to the highest dose of drug (100 mg.kg^{-1}) or equivalent volume of saline (Fig. 24). Administration of the 100 mg.kg^{-1} dose of trientine also increased urine excretion in nondiabetic animals to greater than that of nondiabetic animals receiving the equivalent volume of saline (Fig. 25). This effect was not seen in diabetic animals.

Urinary excretion of Cu and Fe

Analysis of the dose response curves shows that, at all doses, diabetic and nondiabetic animals receiving drug excreted more Cu than animals receiving an equivalent volume of saline (Fig. 26). To provide some correction for the effects of lesser total body growth of the diabetic animals, and thus to allow more appropriate comparison between diabetic and nondiabetic animals, excretion rates of trace elements were also calculated per gram of body weight. Figure 27 shows that diabetic animals had significantly greater copper excretion per gram of body weight in response to each dose of

drug than did nondiabetic animals. The same pattern was seen in response to saline, however the effect was not always significant.

Total copper excreted over the entire duration of the experiment was significantly increased in both nondiabetic and diabetic animals administered trientine compared with their respective saline controls (Fig. 28). Diabetic animals receiving drug also excreted more total copper per gram of body weight than nondiabetic animals receiving drug. A similar, but not significant trend was seen in response to saline administration (Fig. 29).

In comparison, iron excretion in both diabetic and nondiabetic animals receiving trientine was not greater than animals receiving an equivalent volume of saline (Fig. 30). Analysis per gram of body weight shows diabetic animals receiving saline excrete significantly more iron than nondiabetic animals, however this trend was not evident between diabetic and nondiabetic animals receiving trientine (Fig. 31). Total iron excretion in both diabetic and nondiabetic animals receiving drug was not different from animals receiving saline (Fig 32). In agreement with analysis of dose response curves, total iron excretion per gram of body weight was significantly greater in diabetic animals receiving saline than nondiabetic animals but this difference was not seen in response to trientine (Fig 33).

Serum content and renal clearance of Cu and Fe (Table 5)

While there was no significant difference in serum copper content, there was a significant increase in renal clearance of copper in diabetic animals receiving drug compared with diabetic animals receiving saline. The same pattern was seen in nondiabetic animals, although the trend was not statistically significant ($P = 0.056$). There was no effect of drug or state (diabetic versus nondiabetic) on serum content or renal clearance of iron.

Table 5 : Serum content and renal clearance of Cu and Fe in diabetic and nondiabetic animals receiving drug or saline.

	Diabetic		Nondiabetic	
	trientine $n = 6$	*Saline* $n = 7$	*trientine* $n = 4$	*Saline* $n = 7$
Serum Cu ($\mu g.\mu l^{-1} \times 10^{-4}$)	7.56 ± 0.06	9.07 ± 1.74	7.11 ± 0.41	7.56 ± 0.62
Serum Fe ($\mu g.\mu l^{-1} \times 10^{-4}$)	35.7 ± 7.98	63.2 ± 16.4	33.6 ± 1.62	31.4 ± 8.17
Renal clearance Cu ($\mu l.min^{-1}$)	*28.5 ± 4.8	1.66 ± 0.82	5.8 ± 0.28	19.9 ± 6.4
Renal clearance Fe ($\mu l.min^{-1}$)	0.25 ± 0.07	0.38 ± 0.15	0.46 ± 0.22	0.11 ± 0.03

Values shown as mean ± SEM. Asterisk indicates a significant difference ($P < 0.05$) between diabetic animals receiving trientine and diabetic animals receiving an equivalent volume of saline.

Metal content of cardiac tissue (Table6)

Wet heart weights in diabetic animals were significantly less than those in nondiabetic animals while heart/body weight ratios were increased. In some animals cardiac tissue was also analysed for Cu and Fe content. There was no significant difference in content of either metal between diabetic and nondiabetic animals.

Table 6: Heart weight, heart weight/body weight ratios and trace metal content of heart tissue in diabetic versus nondiabetic animals.

	Diabetic	Nondiabetic
Wet heart weight	*0.78 ± 0.02 g	1.00 ± 0.02 g
Heart weight/body weight	*2.93 ± 0.05 $mg.g^{-1}$	2.30 ± 0.03 $mg.g^{-1}$
Cu content	26.7 ± 1.4 $\mu g.g^{-1}$ dry tissue	27.9 ± 0.7 $\mu g.g^{-1}$ dry tissue
Fe content	296 ± 15 $\mu g.g^{-1}$ dry tissue	299 ± 9 $\mu g.g^{-1}$ dry tissue

Diabetic animals: n = 5; nondiabetic animals: n = 10. Values shown as mean ± SEM. Asterisk indicates a significant difference ($P < 0.05$).

THE EFFICACY OF TRIENTINE TO RESTORE CARDIAC FUNCTION IN STZ DIABETIC RATS

INTRODUCTION

Increased tissue copper has been implicated in mechanisms leading to diabetic nerve damage. We therefore hypothesized that tissue accumulation of trace metals might play a role in the mechanisms of diabetic damage in other tissues. Evidence from our earlier studies showed that 6 months of treatment with trientine appears to protect the hearts of diabetic Wistar rats from development of cardiac damage (diabetic cardiomyopathy), as judged by histology. However, it was unknown whether this histological improvement translates into an improvement in cardiac function, a finding that would lend further support to use of trientine therapy in clinical applications.

AIM

The aim of this study was to use an isolated-working-heart model to compare cardiac function in trientine-treated STZ diabetic rats with that in untreated STZ diabetic rats and non-diabetic control rats.

METHODS

Animals

The animals used in these experiments received care that complied with the "Principles of Laboratory Animal Care" (National Society for Medical Research), and the study was approved by the University of Auckland Animal Ethics Committee.

Male albino Wistar rats weighing 330-430g were assigned to three experimental groups as follows:

Table 7 : Experimental groups

Group	Code	N	Treatment
Group A	STZ	8	Diabetes for 13 weeks
Group B	STZ/D6	8	Diabetes for 13 weeks Drug therapy week 7-13
Group C	Sham	9	Non-diabetic controls

STZ = Streptozotocin
D6 = trientine treatment for 6 consecutive weeks

Diabetes was induced by intravenous streptozotocin (STZ; Sigma; St. Louis, MO). All rats were given a short inhalational anaesthetic (Induction: 5% halothane and 2 L/min oxygen, maintained on 2% halothane and 2 L/min oxygen). Those in the two diabetic groups then received a single intravenous dose of STZ (57 mg/kg body weight) in 0.5 ml saline (0.9%) administered via a tail vein. Non-diabetic sham-treated animals received an equivalent volume of saline. Diabetic and non-diabetic rats were housed in like-pairs and provided with free access to normal rat chow (Diet 86 pellets; New Zealand Stock Feeds, Auckland, NZ) and deionized water *ad libitum*. Animals were housed at 21 degrees and 60% humidity in standard rat cages with a sawdust floor that was changed daily.

Blood glucose was measured in tail-tip capillary blood samples (Advantage II, Roche Diagnostics, NZ Ltd). Sampling was performed on all groups at the same time of the day. Blood glucose and body weight were measured on day 3 following STZ/saline injection and then weekly throughout the study. Diabetes was confirmed by presence of polydipsia, polyuria and hyperglycemia (>11 $mmol.L^{-1}$).

In the drug treated diabetic group, trientine was prepared in the drinking water for each cage to a concentration of 50 mg/ml. Animals consumed about 260 ml water per day

once diabetes was established, to yield a total drug dose per day of 13.3 mg/kg. The drug-containing drinking water was administered continuously from week 7 until the animal was sacrificed at week 13. At week 7 animals are expected to have established cardiomyopathy, as shown by our preliminary studies and confirmed in the literature [Rodrigues, 1986 #98].

On the last day of the experiment, animals were anesthetized (5% halothane and 2 $L.min^{-1}$ O_2), and heparin (500 $IU.kg^{-1}$) (Weddel Pharmaceutical Ltd., London) administered intravenously via tail vein. A 2 ml blood sample was then taken from the inferior vena cava and the heart was then rapidly excised and immersed in ice-cold Krebs-Henseleit bicarbonate buffer to arrest contractile activity. Hearts were then placed in the isolated perfused working heart apparatus.

Perfusion

The aortic root of the heart was immediately ligated to the aortic cannula. Retrograde (Langendorff) perfusion at a hydrostatic pressure of 100 cm H_2O and at 37°C was established and continued for 5 min while cannulation of the left atrium via the pulmonary vein was completed. The non-working (Langendorff) preparation was then converted to the working heart model by switching the supply of perfusate buffer from the aorta to the left atrium at a filling pressure of 10 cm H_2O. The left ventricle spontaneously ejected into the aortic cannula against a hydrostatic pressure (afterload) of 76 cmH_2O (55.9 mmHg). The perfusion solution was Krebs-Henseleit bicarbonate buffer (mM: KCl 4.7, $CaCl_2$ 2.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, NaCl 118, and $NaHCO_3$ 25), pH 7.4 containing 11 mM glucose and it was continuously gassed with 95% O_2:5% CO_2. Buffer was continuously filtered in-line (initial 8μm, following 0.4μm cellulose acetate filters; Sartorius, Germany). The temperature of the entire perfusion apparatus was maintained by water jackets; buffer temperature was continuously monitored and adjusted to maintain hearts at 37°C throughout perfusion.

Pressure monitoring and pacing

A modified 24g plastic cannula (Becton Dickson, Utah, USA) was inserted into the left ventricle via the apex of the heart using the normal introducer-needle. This was

attached to a SP844 piezo-electric pressure transducer (AD Instruments) to continuously monitor left ventricular pressure. Aortic pressure was continuously monitored through the side arm of the aortic cannula with a pressure transducer (Statham Model P23XL, Gould Inc., CA, USA). The heart was paced (Digitimer Ltd, Heredfordshire, England) at a rate of 300 bpm by means of electrodes attached to the aortic and pulmonary vein cannulae using supra-threshold voltages with pulses of 5-ms duration from the square wave generator.

Aortic flow measurements

Aortic flow was recorded by an in-line flow meter (Transonic T206, Ithaca, NY, USA) and coronary flow was measured by timed 30sec collection of the coronary vein effluent at each time point step of the protocol.

Working heart apparatus

This working heart apparatus was a variant of that originally described by Neely *et al*, 1967. Our modified apparatus allowed measurements of cardiac function at different preload pressures (Figure 23A and Figure 23B). This was achieved by constructing the apparatus so that the inflow height of the buffer coming to the heart could be altered through a series of graduated steps in a reproducible manner. As in the case of the afterload, the outflow tubing from the aorta could be increased in height to provide a series of defined afterload pressures; these have been converted to mmHg for presentation in the results.

Data collection

All data from the pressure transducers and flow probe were collected (Powerlab 16s data acquisition machine; AD Instruments, Australia). The data processing functions of this device were used to calculate the first derivative of the two pressure waves (ventricular and aortic). The final cardiac function data reported comprised:

Cardiac output*; aortic flow; coronary flow; mean left ventricular pressure developed (MLVDP); maximum rate of ventricular pressure development (+dP/dt)**;

maximum rate of ventricular pressure relaxation (-dP/dt)**; maximum rate of aortic pressure development (aortic +dP/dt); maximum rate of aortic relaxation (aortic -dP/dt). [*Cardiac output (CO) is the amount of buffer pumped per unit time by the heart and is comprised of buffer that is pumped out of the aorta as well as the buffer pumped into the coronary vessels. This is an overall indicator or cardiac function.

** +dP/dt is the rate of change of ventricular (or aortic pressure) and correlates well with the strength of the contraction of the ventricle (contractility). It can be used to compare contractile abilities of different hearts when at the same preload (Textbook of Medical Physiology, Ed. A.Guyton. Saunders company 1986). -dP/dt is an accepted measurement of the rate of relaxation of the ventricle].

Protocol

The experiment was divided into two parts, the first with fixed afterload and variable preload the second, which immediately followed on from the first, with fixed preload and variable afterload.

I) Fixed Afterload and changing Preload

After the initial cannulation was completed, the heart was initially allowed to equilibrate for 6 min at 10 cm of water atrial filling pressure and 76 cm water afterload. During this period the left ventricular pressure transducer cannula was inserted and the pacing unit started. Once the heart was stable, the atrial filling pressure was then reduced to 5 cm H_2O of water and then progressively increased in steps of 2.5 cmH_2O over a series of 7 steps to a maximum of 20 cmH_2O. The preload was kept at each filling pressure for 2min, during which time the pressure trace could be observed to stabilize and the coronary flow was measured. On completion of the variable preload experiment, the variable afterload experiment was immediately commenced.

II) Fixed Preload and changing Afterload

During this part of the experiment the filling pressure (preload) was set at 10 cm H_2O and the afterload was then increased from 76 cm H_2O (55.9 mmHg) in steps of 8 cm

H_2O (5.88 mmHg); again each step was of 2 min duration. The maximum height (afterload) to which each individual heart was ultimately exposed, was determined either by attainment of the maximal available afterload height of 145 cm H_2O (106.66 mmHg), or the height at which measured aortic flow became less than 5ml/min. In the later situation, the heart was considered to have "functionally failed". To ensure that this failure was mechanical and not due to other causes (e.g. ischaemic damage) all hearts were then returned to the initial perfusion conditions (preload 10 cm H2O; afterload 75 cm H2O) for 4 minutes to confirm that pump function could be restored.

At the end of this period the hearts were arrested with a retrograde infusion of 0.4 ml of cold KCL (24 mM). The atria and vascular remnants were then excised, the heart blotted dry, and the ventricles incised midway between the apex and atrioventricular sulcus. Measurements of the ventricular wall thickness were then made using a micro-caliper (Absolute Digimatic, Mitutoyo Corp, Japan).

ANALYSIS

Data from the Powerlab was extracted by averaging 1 min intervals from the stable part of the electronic trace generated from each step in the protocol. The results from each group were then combined and analysed for differences between the groups for the various cardiac function parameters (aortic flow, cardiac flow, MLVDP, LV or aortic +/- dP/dt).

RESULTS

Animals

The weights of the animals at the end of the experimental period are shown in Table 8. Because there was a small difference in the initial weights of the animals, a graph of the percentage change in weight for each group has been included (Figure 5B).

Table 8: Initial and Final Animal weights (mean ± SD)

	Number (n)	Treatment	Initial weight (g)		Final weight (g)	
Group A	9	STZ	361 ±12	*	221±27	*
Group B	8	STZ/D6	401 ± 33		290±56	
Group C	8	Sham	361 ±16	*	574±50	

* $P < 0.01$

Diabetic status

Blood glucose values for the three groups of rats are presented in Figure 6. Generally, the presence of diabetes was confirmed established within 3-5 days following the STZ injection. The Sham control group remained normoglycaemic throughout the experiment, and treatment with the drug made no difference to their blood glucose profile (p=ns), as expected.

Heart weights

Final heart weight and ventricular wall thickness measurements are presented in Table 9.

Table 9: Final heart weights (g) and per g of animal body Weight (BW) (mean ± SD)

Group	Heart weight (g)		Heart weight (g) /BW (g)		Left Ventricular wall thickness (mm)		Left Ventricular wall thickness (mm)/BW (g)	
Sham	1.58 ± 0.13§		0.0028±0.0002§		3.89±0.38§		0.0068±0.0009§	
STZ/D6	1.18 ± 0.24	ns	0.0041±0.0005	*	3.79±0.52	ns	0.0127±0.0027	ns
STZ	1.03 ± 0.17		0.0047±0.0004		3.31±0.39		0.0152±0.0026	

- P=0.02
- § = Highly significant with the other 2 groups

Part I results

The following graphs of Figures 7 to 14 demonstrate cardiac performance parameters of the animals (STZ diabetic; STZ diabetic +drug; and sham-treated controls) while undergoing increasing atrial filling pressure (5-20 cmH_2O, preload) with a constant afterload of 75 cm H_2O.

Cardiac output (Figure 7) is the sum to the aortic flow (Figure 9) and the coronary flow as displayed in Figure 8. Since the control hearts and experimental groups have significantly different final weights, the coronary flow is presented (Figure 8A) as the flow normalized to heart weight [Note that coronary flow is generally proportional to cardiac muscle mass, and therefore to cardiac weight].

The mean left ventricular developed pressure (MLVDP) is summarized in Figure 10. The first derivative of the pressure curve gives the rate of change in pressure development in the ventricle with each cardiac cycle and the maximum positive rate of change (+dP/dt) value is plotted in Figure 11. The corresponding maximum rate of relaxation (-dP/dt) is in Figure 12. Similar information appears in Figure 13 and Figure 14, in which the maximum positive and negative rates of change in pressure within the aortic outflow tubing are shown.

All results are mean ± sem.

Sham (n=9), STZ diabetic (n=8), STZ Diabetic + Drug (D6) (n=8)

# $p<0.01$ STZ/D6 v STZ

*$p<0.01$ STZ/D6 v Sham

Part II results

A similar set of graphs are presented in Figures 15 to 22 and Figure 34 which demonstrate equivalent functional outcomes, but this time under conditions of constant preload and changing afterload. Results are mean ± sem.

Note: In part I of the experiment, all hearts remained functional throughout all the changes in preload. However in this section, the increased work associated with the higher afterloads was used as an additional indicator of cardiac function. The numbers of functionally surviving hearts (n) in each group varies at higher afterload levels. This attrition reflects functional failure of the heart at the stated level of afterload. (See Table 10 and Figure 34).

Table 10 : Cardiac survival at each afterload pressure

Number surviving						Percentage			
Afterload (mmHg)	STZ	STZ/D6	Sham			Afterload (mmHg)	STZ	STZ/D6	Sham
55.9	8	8	9			55.9	100%	100%	100%
61.8	8	8	9			61.8	100%	100%	100%
67.7	8	8	9			67.7	100%	100%	100%
71.4	6	8	9			71.4	75%	100%	100%
77.2	5	8	9			77.2	63%	100%	100%
83.1	4	8	9			83.1	50%	100%	100%
88.3	3	7	9			88.3	38%	88%	100%
94.9	1	6	9			94.9	13%	75%	100%
100.8	0	5	9			100.8	0%	63%	100%
106.7	0	1	9			106.7	0%	13%	100%

SUMMARY

- Treatment with trientine had no obvious effect on blood glucose concentrations in the two diabetic groups (as expected).

  There was a small but significant improvement in the cardiac weight /body weight ratio in the trientine-treated diabetic group compared to that of the untreated diabetic group.
- When the coronary flow was normalized to heart weight, the drug treated group still showed improved flows at lower filling pressures than did the untreated diabetic animals.
- Indicators for ventricular contraction and relaxation were both significantly improved in the drug treated group compared to equivalent values in the untreated diabetic group. The improvement restored function to such an extent that there was no significant difference between the drug treated and the sham-treated control groups.

- The aortic transducer measures of pressure change also showed improved function in the drug treated group
- When afterload was increased in the presence of constant preload, it was observed that the heart's ability to function at higher afterload was greatly improved in the drug treated diabetic group compared to the untreated diabetic group. When 50% of the untreated hearts had failed,100% of the drug treated hearts were still functioning.
- Compared to the untreated diabetic hearts, the response of the drug treated diabetic hearts showed significant improvements in several variables: cardiac output, aortic flow, coronary flow, as well as improved ventricular contraction and relaxation indices in both the ventricular and aortic pressure wave profiles.

CONCLUSION

These preliminary data suggest that treatment of STZ diabetic rats with trientine dramatically improves several measures of cardiac function.

Additional relevant notes:

Other published studies in Wistar rats have shown that the functional changes of the cardiomyopathy of STZ rats is

- Cardiomyopathy is present after 5-6 weeks following STZ
  Rodrigues B, Am J Physiol 251 H571-H580 1986
- Not related to heart size: since small weight matched hearts have same function as larger control hearts (we confirmed this in our pilot study as well)
- Not due to malnutrition effects of low insulin: since calorie deprived rats with hearts of the same weight had the same function as larger control hearts
- Not substrate dependent: addition of extra insulin and increased glucose did not reverse the functional deficit
- Probably not due to the STZ itself: since animals given STZ in this study but that failed to go on to develop diabetes, did not get a change in heart function
  Penpargkul S et al, Cir Res 4: 911-921 1980 (Good review and is in the folder)

Interim Conclusions:

Administration of oral Trientine for 6 weeks in Wistar rats with previously established diabetes of 7 weeks duration (A duration known from the literature and our first pilot study, to be associated with significant cardiomyopathy) resulted in a global improvement in cardiac function. This improvement was demonstrated by measures of improved contractile function (LVDP most clearly seen in the afterload experiment +dP/dT) and a reduction in ventricular stiffness (-dP/dT). These parameters improved in the presence of either increasing preload or after-load protocols.

SUMMARY

The acute effect of intravenous trientine administration on urinary excretion of copper and iron was studied in anesthetized, diabetic (6 weeks of diabetes, Streptozotocin induced) and nondiabetic rats. Animals were assigned to one of four groups: diabetic + trientine, diabetic + saline, nondiabetic + trientine, nondiabetic + saline. Drug, or an equivalent volume of saline, was administered hourly in doses of increasing strength (0.1, 1.0, 10, 100 $mg.kg^{-1}$) and urine was collected throughout the experiment in 15 min aliquots. A terminal blood sample was taken and cardiac tissue harvested.

Analysis of urine samples showed the following main points:

- At all drug doses, diabetic and nondiabetic animals receiving drug excreted more Cu (μg) than animals receiving an equivalent volume of saline.
- When analsyed per gram of bodyweight, diabetic animals excreted significantly more copper ($\mu g.gBW^{-1}$) at each dose of trientine than did nondiabetic animals. The same pattern was seen in response to saline but the effect was not significant at every dose.
- At most doses, in diabetic animals iron excretion (μg) was greater in animals administered saline than in those administered drug. In nondiabetic animals there was no difference between iron excretion in response to saline or trientine administration.
- Analysis per gram of body weight shows no difference between iron excretion in nondiabetic and diabetic animals receiving trientine. Diabetic

animals receiving saline excrete more iron per gram of bodyweight than nondiabetic animals receiving saline.

Analysis of heart tissue showed no significant difference in content of either metal between diabetic and nondiabetic animals, nor any effect of drug on cardiac content of iron and copper.

Renal clearance calculations showed a significant increase in clearance of copper in diabetic animals receiving trientine compared with diabetic animals receiving saline. The same trend was seen in nondiabetic animals but the effect was not significant. There was no effect of trientine on renal clearance of iron.

In summary, trientine-treatment effectively increases copper excretion in both diabetic and nondiabetic animals. The excretion of copper in urine following trientine administration, is greater per gram of bodyweight in diabetic than in nondiabetic animals. Iron excretion was not increased by trientine treatment in either diabetic or nondiabetic animals.

Statistical methods

Data for each dose level were analysed using a mixed linear model (PROC MIXED; SAS, Version 8). The model included diabetes, drug and their interaction as fixed effects, time as a repeated measure, and rats as the subjects in the dataset. Complete independence is assumed across subjects. The full model was fitted to each dataset using a maximum likelihood estimation method (REML) fits mixed linear models (i.e., fixed and random effects models). A mixed model is a generalization of the standard linear model, the generalization being that you can analyse data generated from several sources of variation instead of just one. A level of significance of 0.05 was used for all tests

RESULTS

Copper

Diabetic rats excreted significantly higher levels of copper across all dose levels. Baseline copper excretion was also significantly higher in diabetic rats compared to and prior to drug administration. The drug resulted in a significantly higher excretion of copper compared to saline at all does levels. There was no difference at baseline levels between the drug

and saline groups. The interaction effect for the model was significant at dose levels of 1.0 $mg.kg^{-1}$ and above. The presence of a significant interaction term means that the influence of one each effect vary with the level of the other effect. Therefore, the outcome of a significant interaction between the diabetes and drug factors in increased copper excretion above the predicted additive effects of these two factors.

Iron

Diabetic rats in the saline only group excreted significantly higher levels of iron at all dose levels. This resulted in all factors in the model being significant across all dose levels.

Statistical analysis using a mixed linear model.

Data for each dose level were analysed using a mixed linear model (PROC MIXED; SAS, Version 8). The model included *diabetes*, *drug* and their interaction as fixed effects, *time* as a repeated measure, and *rats* as the subjects in the dataset. Complete independence is assumed across subjects. The full model was fitted to each dataset using a maximum likelihood estimation method (REML) fits mixed linear models (i.e., fixed and random effects models). A mixed model is a generalization of the standard linear model, the generalization being that you can analyse data generated from several sources of variation instead of just one. A level of significance of 0.05 was used for all tests.

Results from application of the above mixed linear model to the experimental analysis (Figure 35).

Diabetic rats excreted significantly higher levels of copper across all dose levels. Baseline copper excretion was also significantly higher in diabetic rats compared to and prior to drug administration. The drug resulted in a significantly higher excretion of copper compared to saline at all dose levels. There was no difference at baseline levels between the drug and saline groups. The interaction effect for the model was significant at dose levels of 1.0 $mg.kg^{-1}$ and above. The presence of a significant interaction term means that the influence of one effect varies with the level of the other effect. Therefore, the

outcome of a significant interaction between the diabetes and drug factors is increased copper excretion above the predicted additive effects of these two factors.

Diabetic rats in the saline only group excreted significantly higher levels of iron at all dose levels. This resulted in all factors in the model being significant across all dose levels.

HUMAN STUDIES – Phase II

Table 11 shows baseline information on 30 patients with long-standing type 2 diabetes, no clinical evidence of coronary artery disease and abnormal diastolic function who participated in a 6-month randomised, double blind, placebo controlled study of chronic oral therapy with trientine dihydrochloride.

TABLE 11 : Characteristics of Study Participants

	Placebo	Trientine dihydrochloride
N	15	15
Median age (years)	54 (range 43-64)	52 (range 33-69)
% female	44%	56%
Median duration of diabetes (years)	10 (6-24)	8 (4-15)
Mean body mass index (kg/m^2)	32 (SD 5)	34 (SD 5)
% hypertensive	64%	80%
% HbA_{1c}>8	93%	80%

MRI scans of the heart at baseline and 6-months showed a significant reduction in LV mass and a significant improvement in diastolic function measured as a change in apical rotation (AR) at the end of systole. See, Table 12. These effects indicate improved structure and function in the human heart following 6 months of trientine theapy.

TABLE 12 : Phase II: INFO-Cardiac MRI Results

	Placebo (n=15)	GC811007 (n=15)	P
Baseline LVM	202.17	207.45	0.778
Δ LVM 1-6mo	+6.57	-10.49	0.0045
Baseline AR	12.37	12.49	0.931
Δ AR 1-6mo	+0.81	-2.19	0.029

Therefore, an equivalent dose of oral trientine dihydrochloride corrected for weight (15 mg/kg) is effective in both rats and humans.

Compared with non-diabetic animals, STZ diabetic rats show a 2-fold to 10-fold excess of urinary copper excretion following parenteral administration of trientine hydrochloride. Cupuresis is rapid with excess urinary copper excretion manifest within the first 15 minutes of an intravenous bolus injection. Trientine dihydrochloride is effective by parenteral injection in a 10-fold to 100-fold lower dose than the current oral therapy. See, Figure 3 and our patent application filed simultaneously herewith directed to slow release and low dosage forms and their use.

An equivalent dose of oral trientine dihydrochloride corrected for weight (15mg/kg) is effective in both rats and humans.

DOSING REGIMEN FOR TRIENTINE

The half life of trientine, indicated for the treatment and reversal of heart failure and coronary heart disease, is relatively short – being approximately 2 hours. To maintain optimal blood levels, either multiple dose regimen, or a controlled release preparation requiring fewer doses per day is required.

With reference to Figures 36 and 37 there is shown the plasma concentration – time profiles of trientine after oral administration. The plasma concentration was determined using the process as defined in Miyazaki, K., et al., Determination of trientine in plasma

of patients with high-performance liquid chromatography. Chem Pharm Bull, 1990. 38:p. 1035-38.

Ideally trientine should be taken in addition to current therapies, at a maximum tolerated dose, utilizing a dose regimen which fits its pharmacokinetic profile. Patients with heart failure and/or coronary artery disease are frequently on multiple drug regimens. Therefore, a controlled release preparation requiring fewer doses per day is preferred. The proof of principle Phase 2 study has shown positive results. However, the dosage regimen was sub-optimal when compared with its pharmacokinetic profile and the study does not assure the efficacy of the drug which would be required in pivotal trials by regulatory authorities.

For these purposes, the crystalline trientine dihydrochloride salt may be administered parenterally (including subcutaneous injections, intravenous, intramuscular, intradermal injection or infusion techniques) or by inhalation spray in dosage unit formulations containing conventional non-toxic pharmaceutically-acceptable carriers, adjuvants and vehicles.

Routes for parenteral administration therefore include intravenous, intramuscular, intraperitoneal, subdermal, and subcutaneous administration.

The injectable solutions or suspensions may be formulated according to known art, using suitable non-toxic, parenterally-acceptable diluents or solvents, such as mannitol, 1,3-butanediol, water, Ringer's solution or isotonic sodium chloride solution, and/or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono-or diglycerides, and fatty acids, including oleic acid.

Suitable dispersing or suspending agents for aqueous suspensions include synthetic and natural gums such as tragacanth, acacia, alginate, dextran, sodium carboxymethylcellulose, methylcellulose, polyvinyl-pyrrolidone or gelatin.

A formulation for injection, infusions, etc. may include such preservatives and other inclusions desired having regard to shelf life requirements and mode of administration.

The pharmaceutical preparation may also contain non-toxic auxillary substances such as antibacterial components for example quaternary ammonium compounds, known

to have cold sterilizing properties and which are non-injurious in use, such as thimerosal, methyl and propyl paraben, benzyl alcohol, phenyl ethanol.

Other suitable additions to the injectable solution include antibacterial preservatives, buffers, solubilizers, antioxidants, and other pharmaceutical adjuncts.

Extended-release formulations containing trientine or salts thereof suitable for parenteral administration. Extended rates of drug action following injection may be achieved in a number of ways, including the following: crystal or amorphous drug forms having prolonged dissolution characteristics; slowly dissolving chemical complexes of the drug entity; solutions or suspensions of drug in slowly absorbed carriers or vehicles (as oleaginous); increased particle size of drug in suspension; or, by injection of slowly eroding microspheres of drug (for example, see: Friess, W., Lee, G. and Groves, M. J. Insoluble collagen matrices for prolonged delivery of proteins. Pharmaceut Dev Technol 1996;1:185-193). The duration of action of the various forms of insulin for example is based in part on its physical form (amorphous or crystalline), complex formation with added agents, and its dosage form (solution of suspension).

In addition to the above means of achieving extended drug action, the rate and duration of drug delivery may be controlled by slow intravenous or subcutaneous infusion, using mechanically-controlled drug infusion pumps. Reference is made herein to our simultaneous filing on the slow release and/or other related means of administration as defined in that patent specification.

Further examples of parenteral products with long-acting properties are available from the USP (supra).

Yet further embodiments of the invention include the incorporation of trientine or its salts into suppositories (rectal or vaginal), or into vaginal inserts.

The injectable formulation may be a suspension and can be prepared by reducing the active agent to a very fine powder with a ball mill, micronizer, colloid mill, or other appropriate equipment and then suspending the material in a liquid in which it is insoluble.

Alternatively the injection can be packaged as a dry solid rather than in conjunction with a solvent or vehicle due to the instability of the therapeutic agent in the presence of

the liquid component, and that upon addition of suitable vehicle(s), yield solutions or suspensions conforming in all respects to the requirements for injection.

In other forms the active ingredient is packaged as a dry powder in conjunction with the liquid material for use at the time of reconstitution.

A suitable injectable formulation, particularly where mixed from crystals of trientine dihydrochloride, sterile water, a phosphate buffering system (eg to pH 7.4 +/- 0.1) and optionally any other desirable excipients, diluent, adjuvants, all as indicated in the art as useful in parenteral formulations. For example a preferred method of preparing a formulation for parenteral administration may be prepared by dissolution of trientine hydrochloride in water and dilution out to 154mM in phosphate buffered saline.

Whilst other buffering systems may be used preferably all formulations are aqueous based.

DATED THIS 20th DAY OF August 200[illegible]
AJ PARK
PER
AGENTS FOR THE APPLICANT

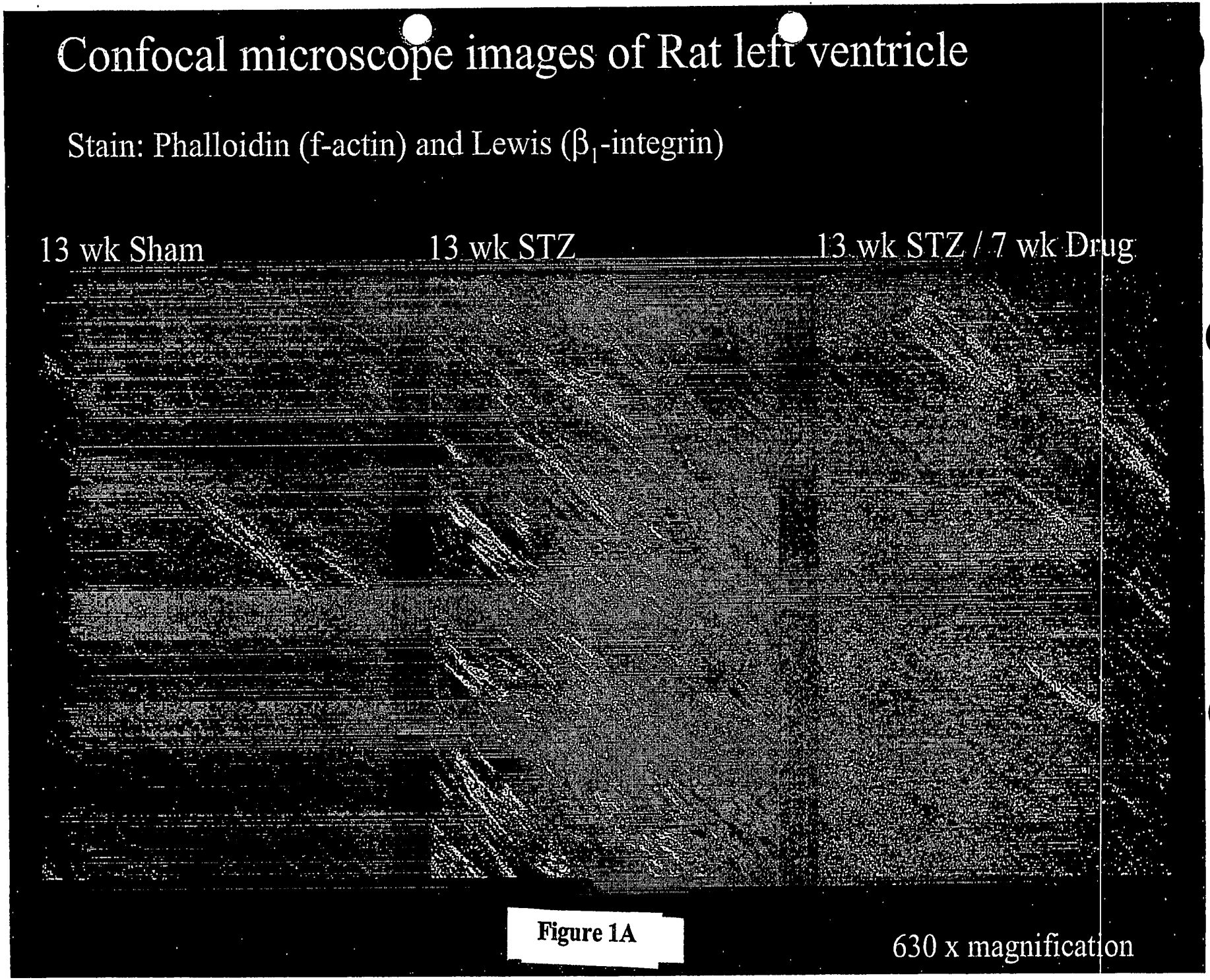

Confocal microscope images of Rat left ventricle
Stain: Phalloidin (f-actin) and Lewis (β_1-integrin)
13 wk Sham
13 wk STZ
13 wk STZ / 7 wk Drug
630 x magnification

Figure 1A

Confocal microscope images of Rat left ventricle

Stain: Phalloidin (f-actin) and Lewis (β_1-integrin)

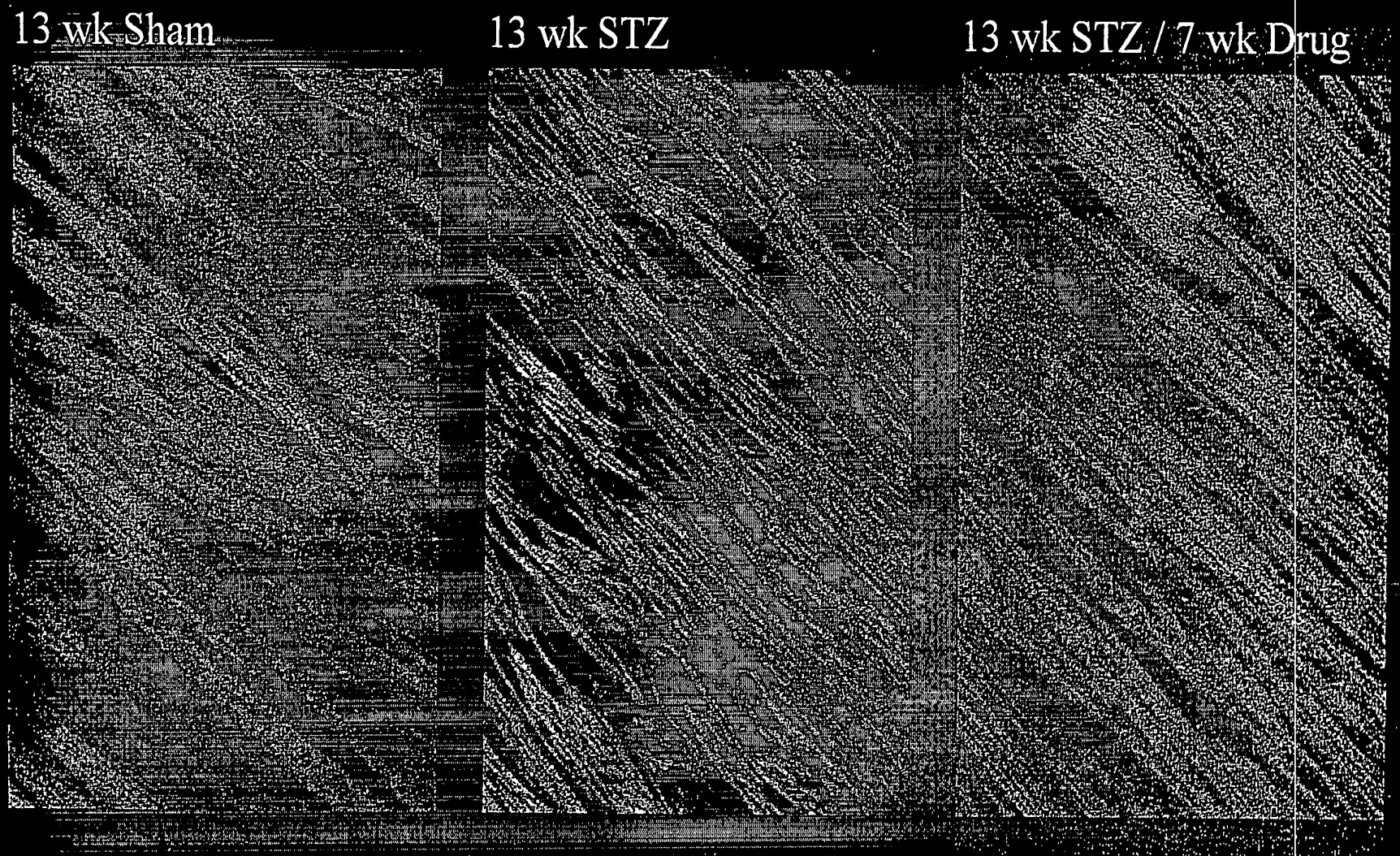
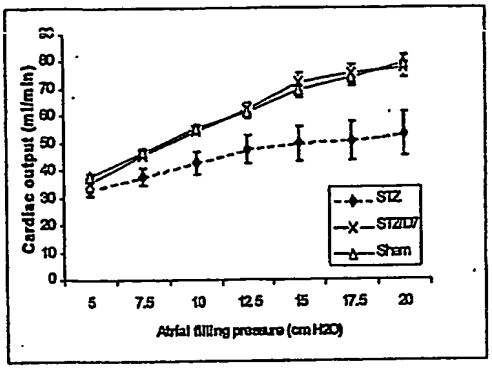


Figure 1B

630 x magnification

Trientine Restores Normal Heart Function



Cardiac output in response to increasing preload with GC811007 treatment from weeks 7-13

STZ/D7 v STZ = $p<0.03$; STZ/D7 v Sham = ns; STZ v Sham = all $p<0.05$

FIGURE 2A

Functional Survival of Working Hearts

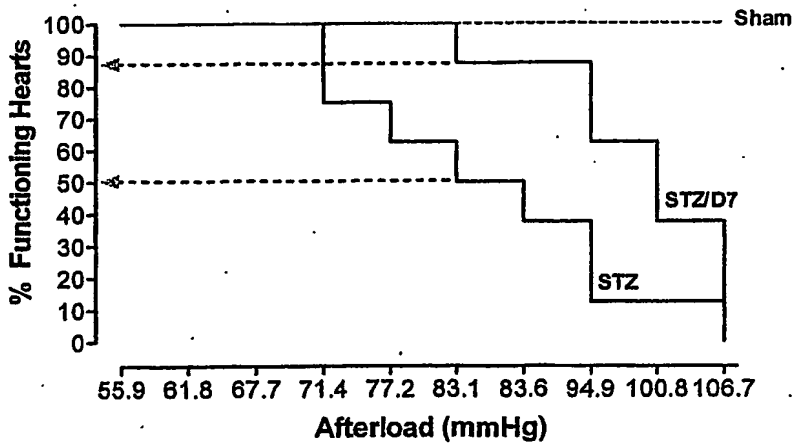
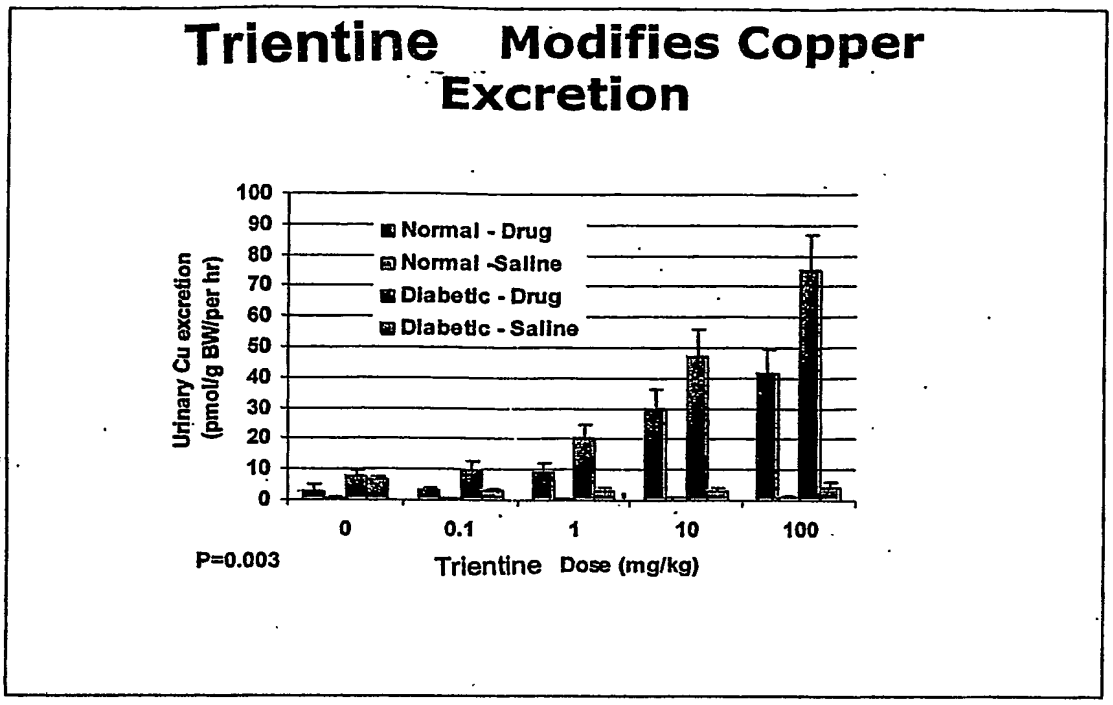


FIGURE 2B



No effect on iron excretion

Indicate where human dose is on x axis

FIGURE 3

Figure 4 Absolute Weight Change with time

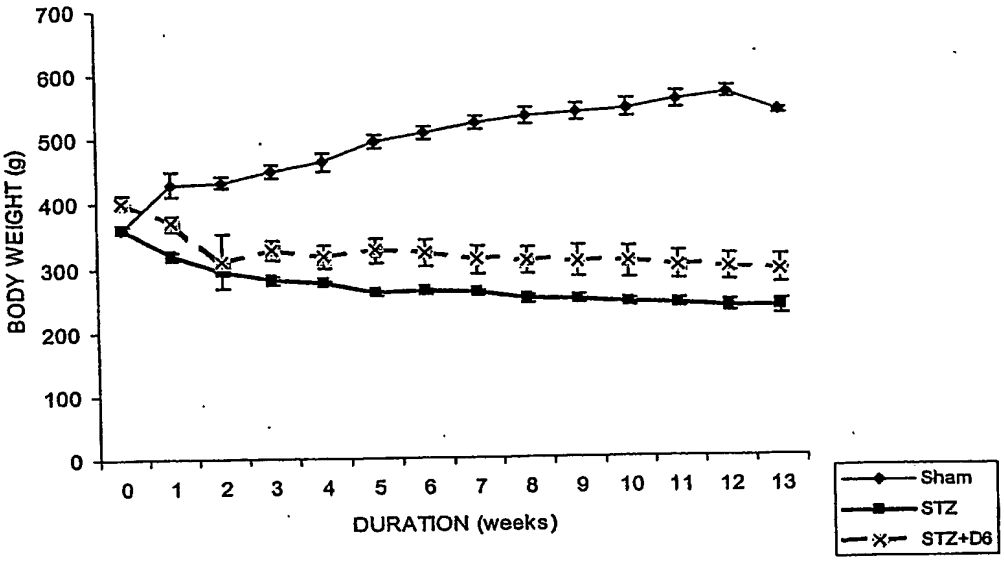


Figure 5 Percentage change in weight over time

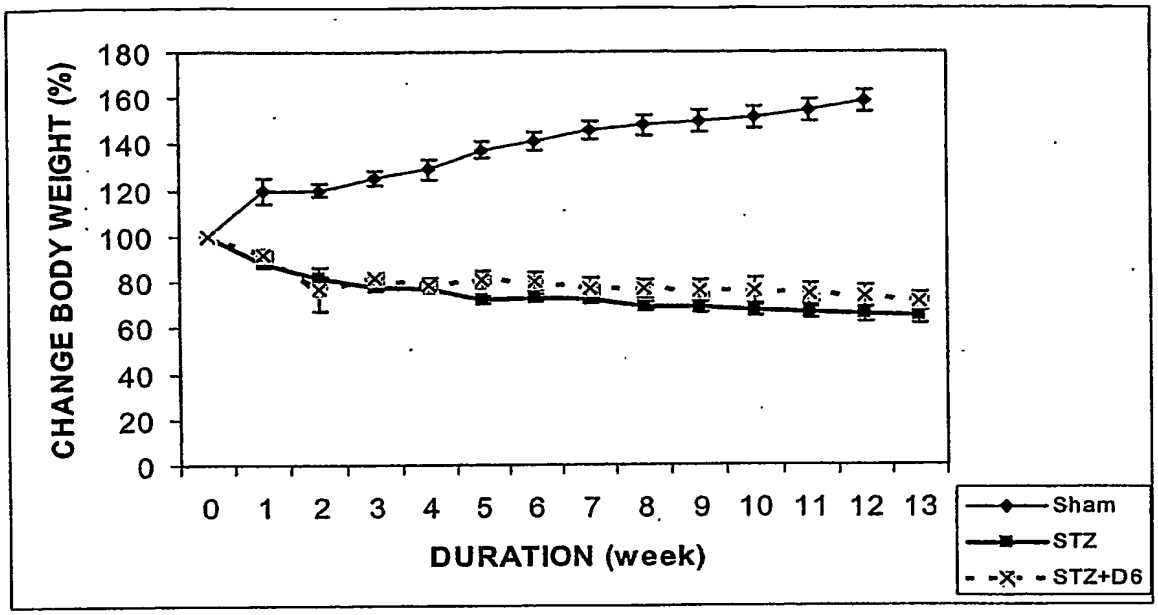


Figure 6 Blood Glucose change over time

(Weekly glucose measurements)

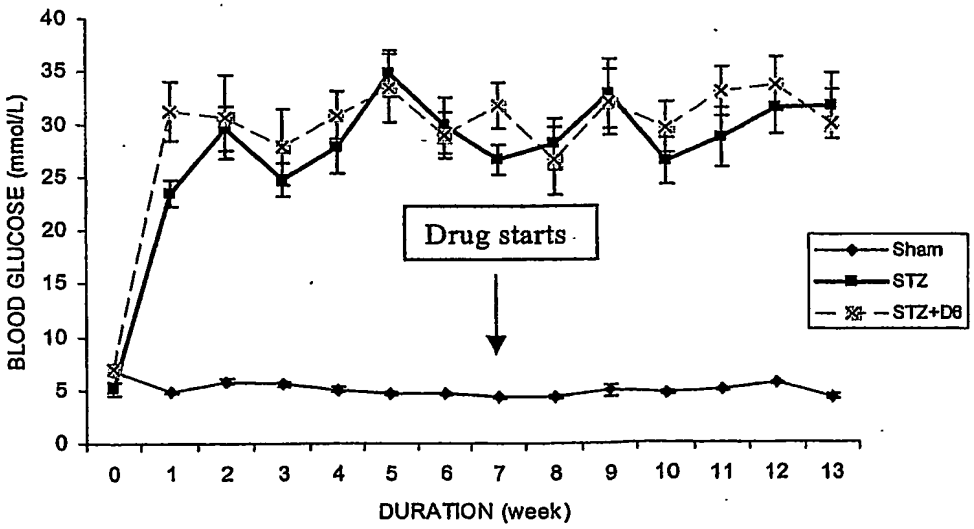


Figure 7 Cardiac output in response to increasing preload

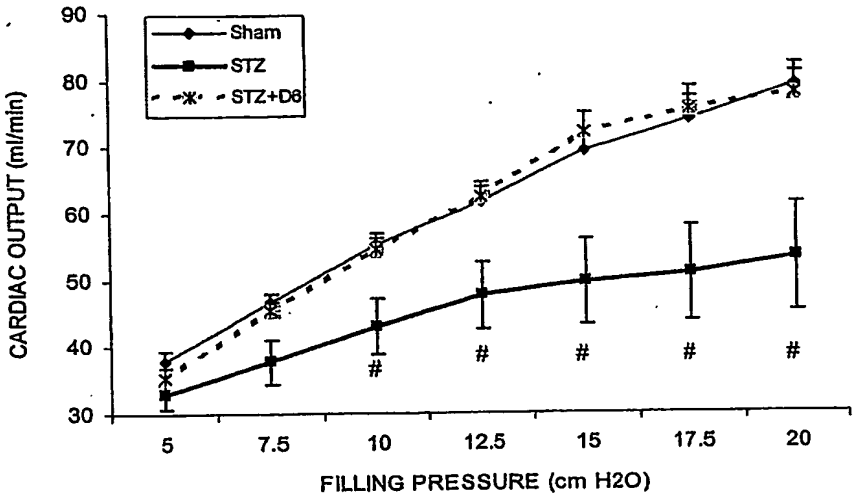


Figure 8. Absolute coronary flow in response to increasing preload

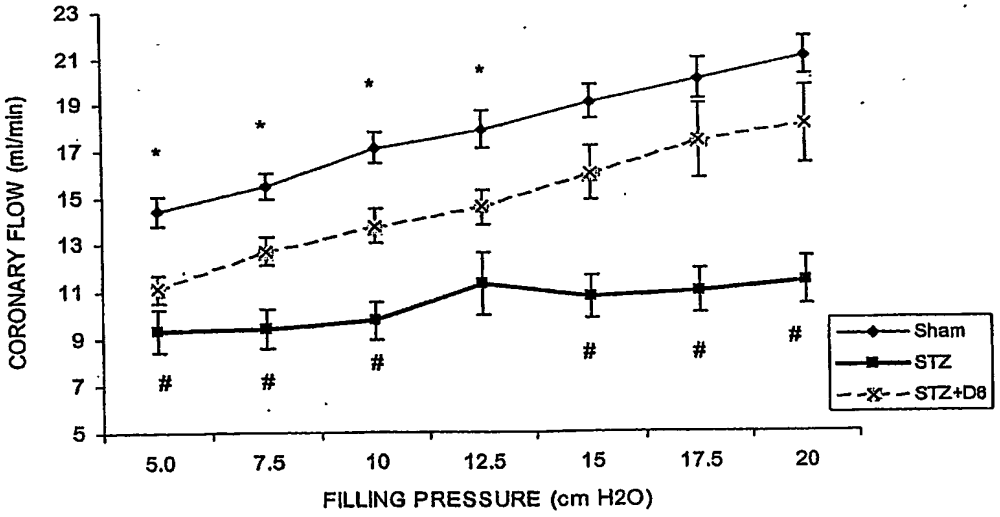


Figure 8A Coronary flow normalized to final cardiac weight

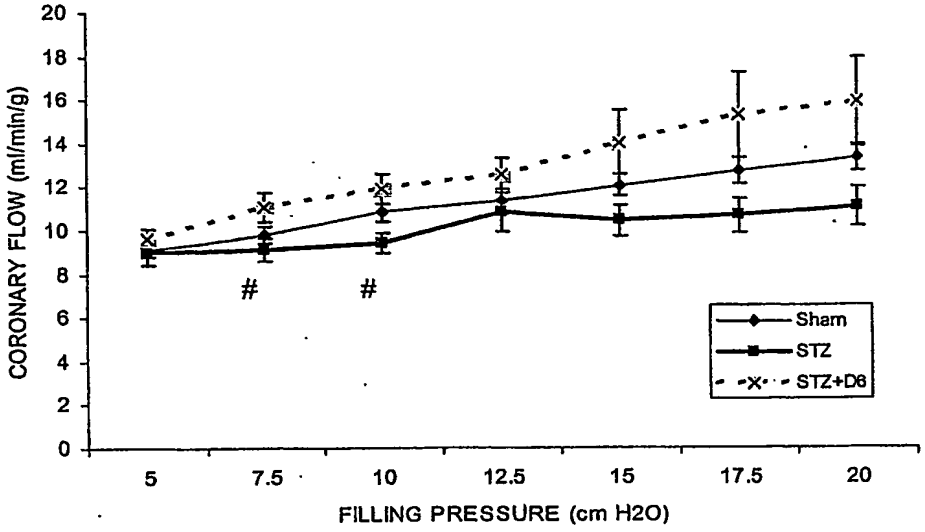


Figure 9 Aortic flow with increasing preload

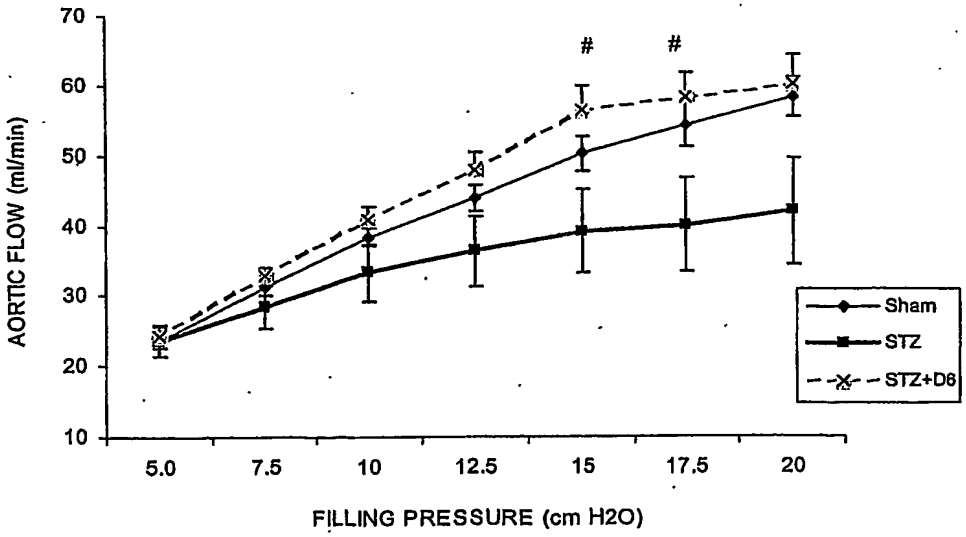


Figure 10 Mean left ventricular developed pressure (MLVDP) in response to increasing preload

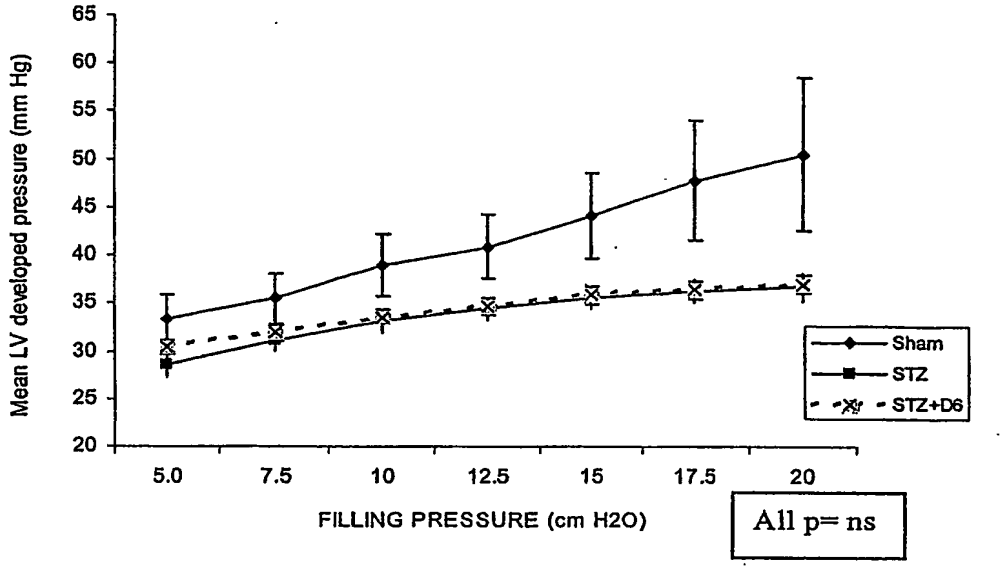


Figure 11 Maximal rate of positive change in ventricular pressure (contraction) in response to increasing preload

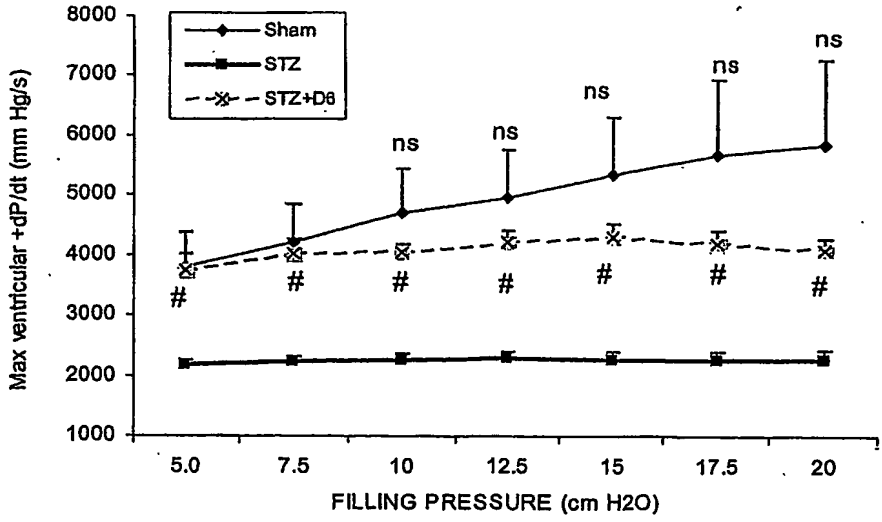


Figure 12 Maximal rate of decrease in ventricular pressure (relaxation) in response to increasing preload

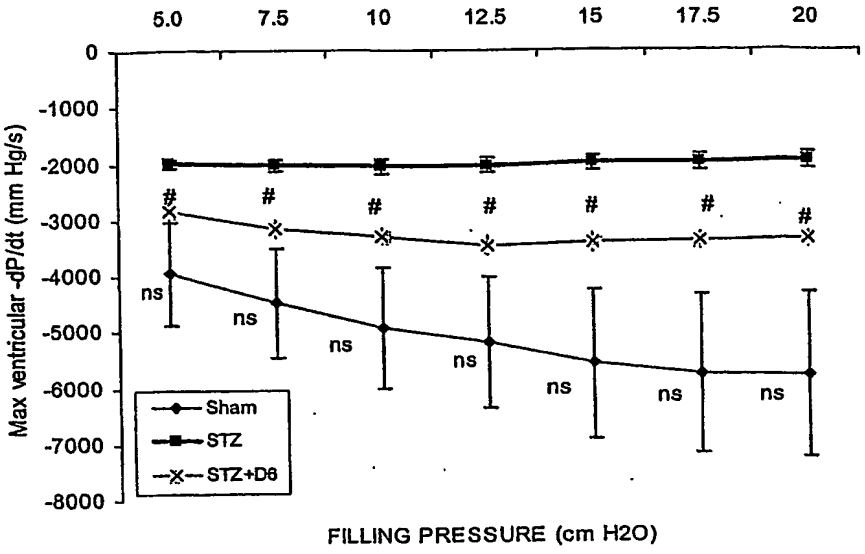


Figure 13 Maximal rate of positive change in aortic pressure in response to increasing preload

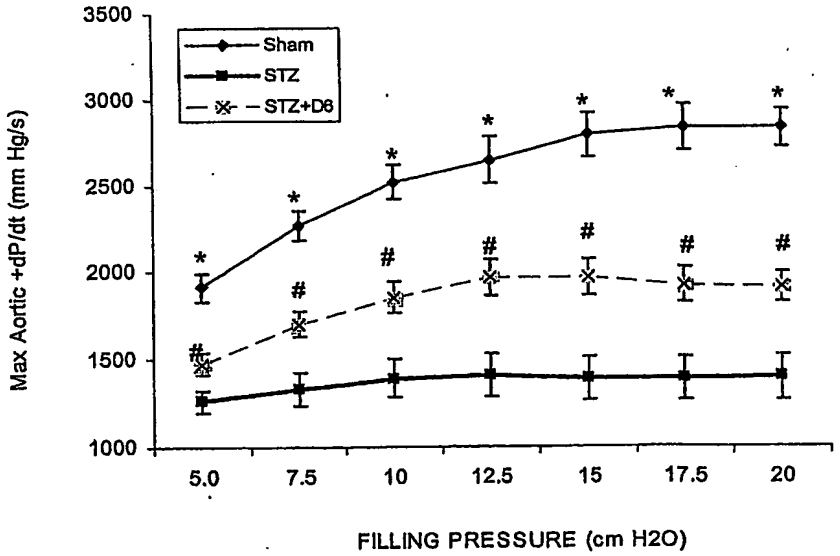


Figure 14 Maximal rate of decrease in aortic pressure in response to increasing preload

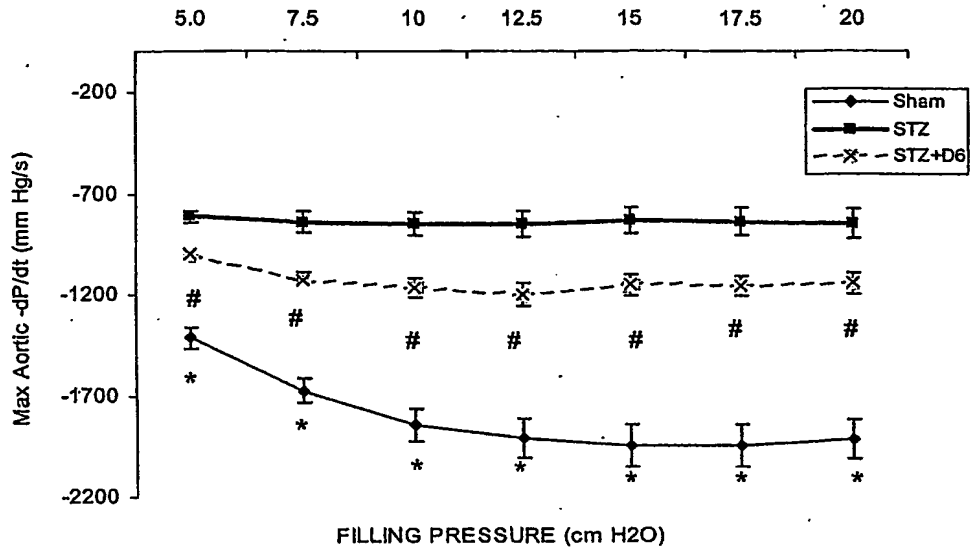


Figure 15 Percentage of functionally surviving hearts at each afterload pressure

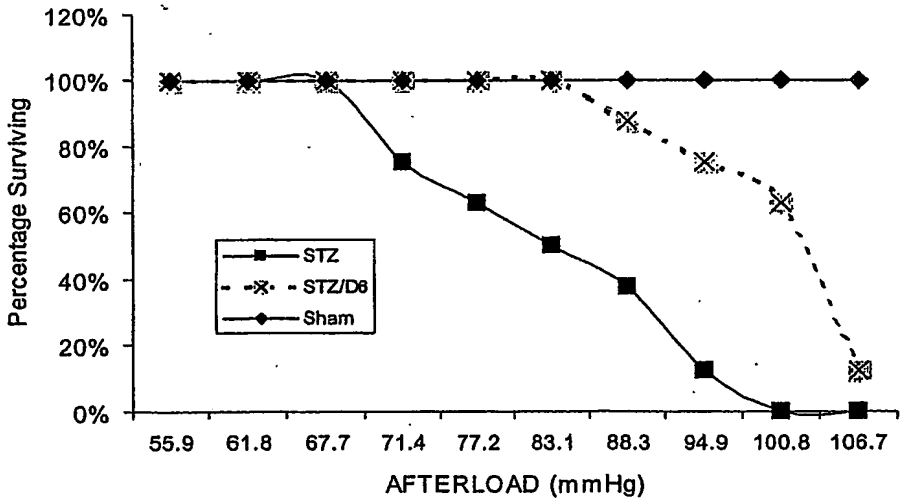


Figure 16 Cardiac output in response to increasing afterload

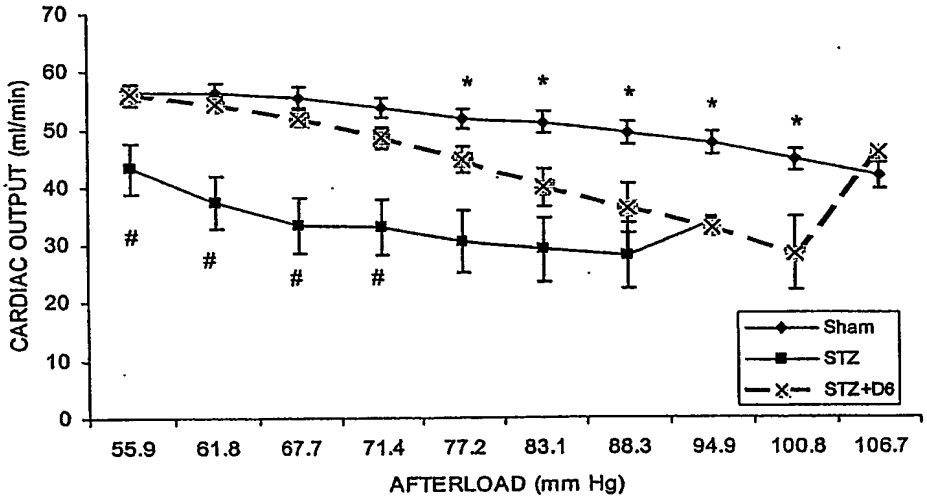


Figure 17A Absolute change in coronary flow in response to increasing afterload

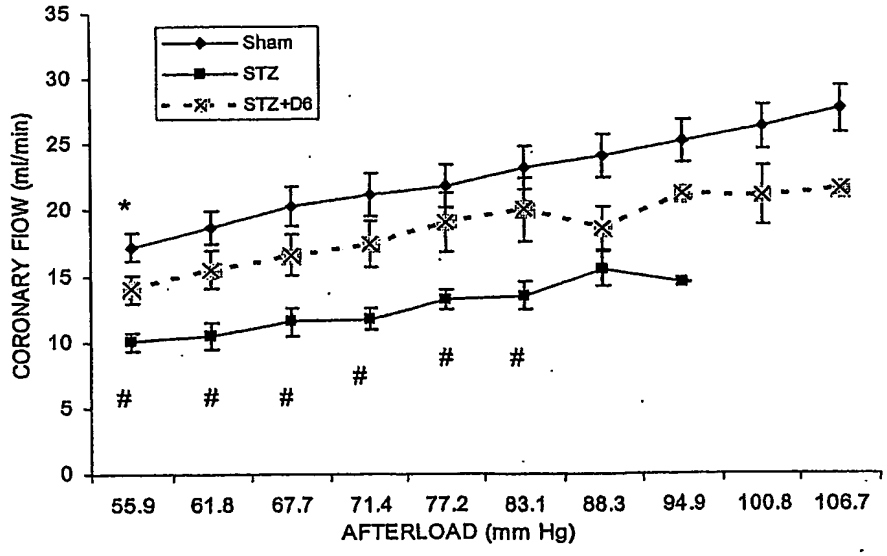


Figure 17B Change in coronary flow in response to increasing afterload normalized to heart weight

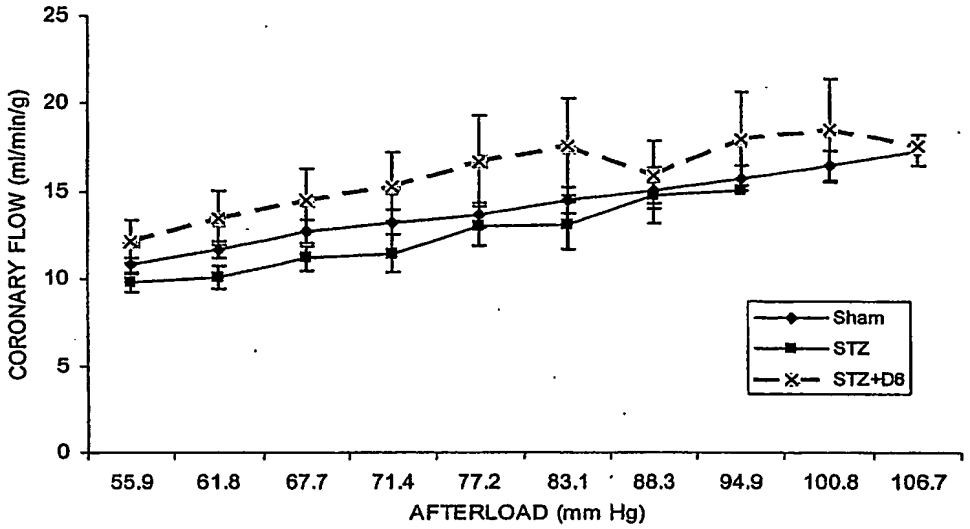


Figure 18 Mean left ventricular developed pressure (MLVDP) in response to increasing afterload

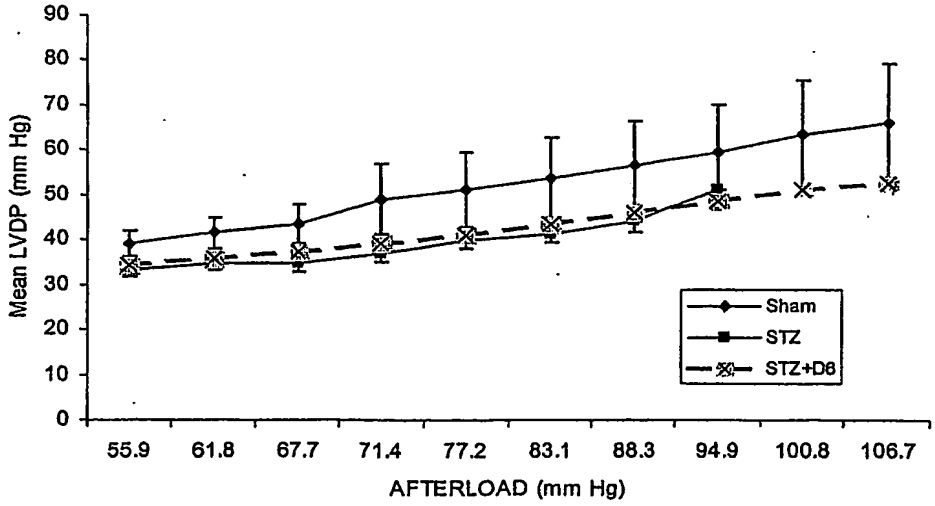


Figure 19. Maximal rate of positive change in ventricular pressure (contraction) in response to increasing afterload

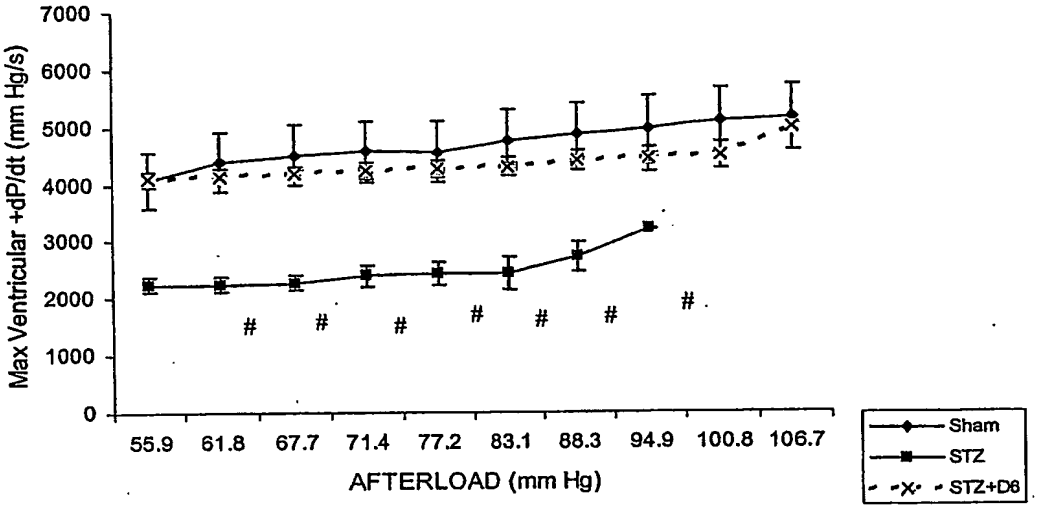


Figure 20. Maximal rate of decrease in ventricular pressure (relaxation) in response to increasing afterload

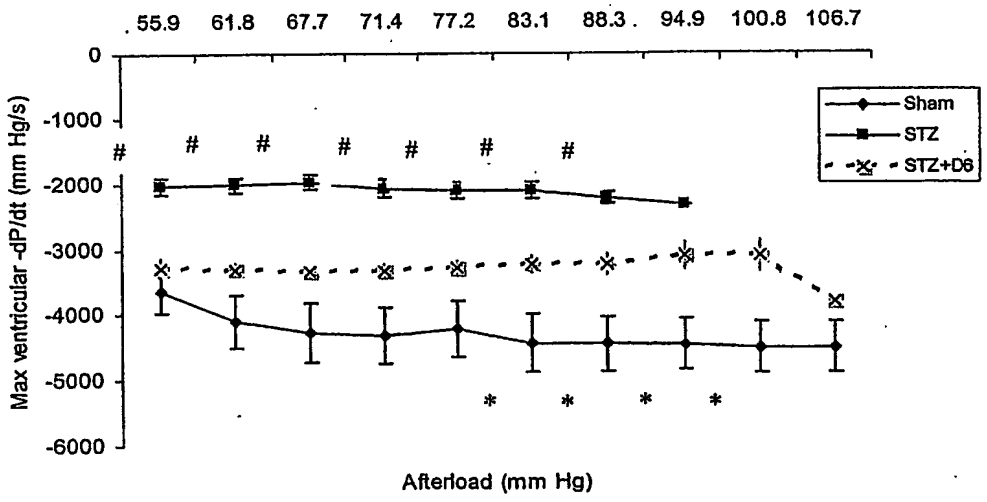


Figure 21 Maximal rate of positive change in aortic pressure in response to increasing afterload

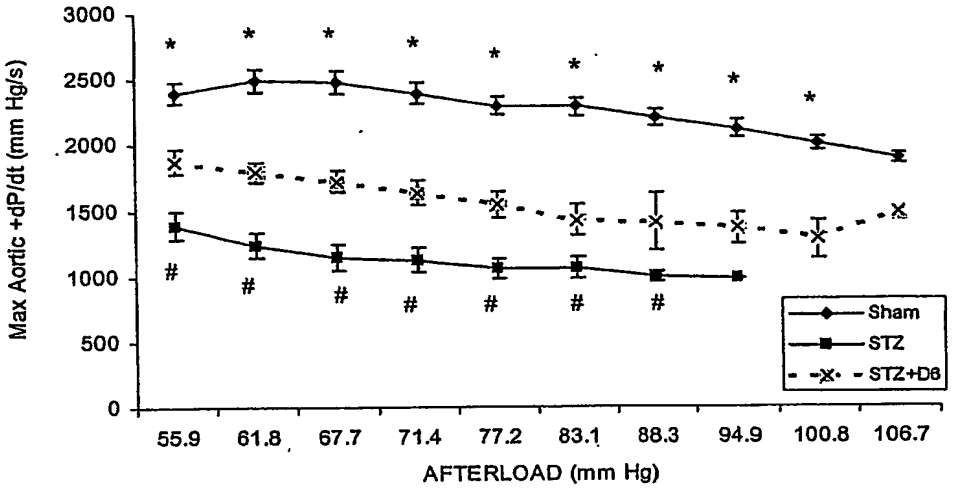


Figure 22. Maximal rate of decrease in aortic pressure in response to increasing afterload

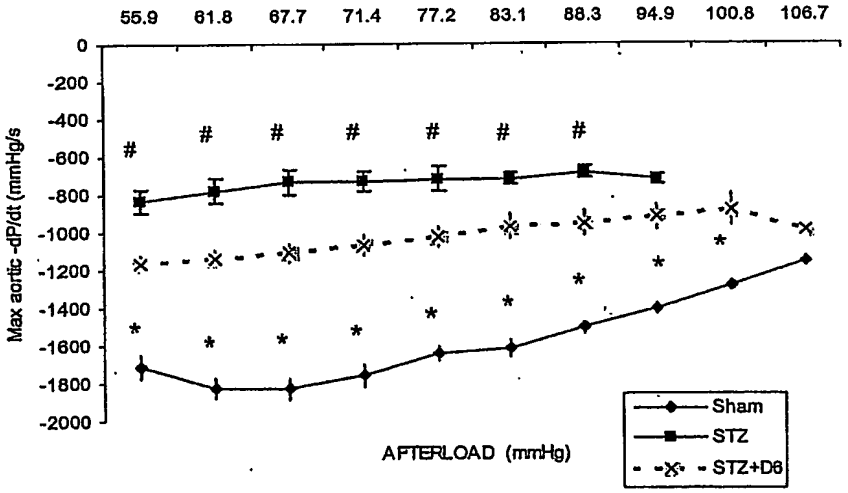
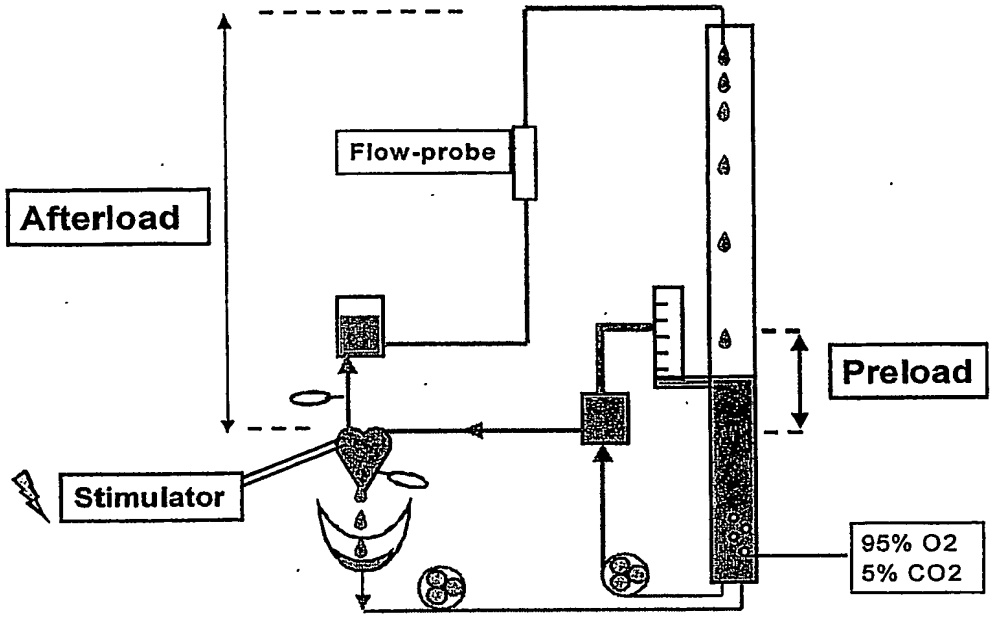
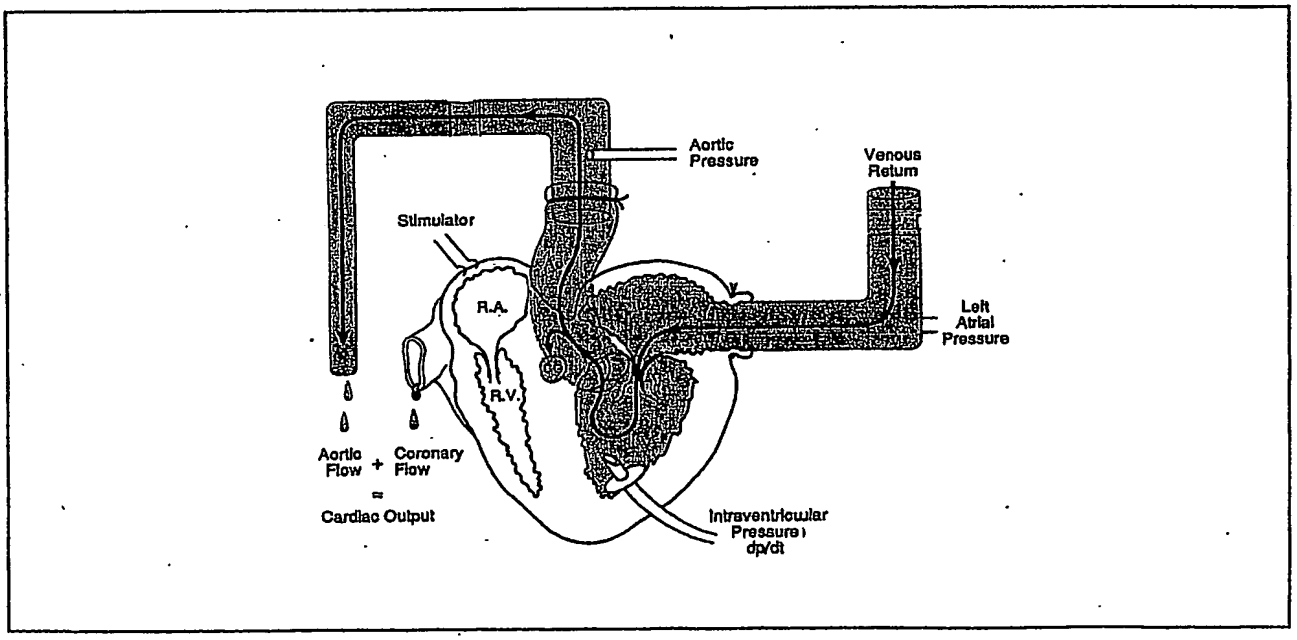


Figure 23A Apparatus for working heart



Pressure transducers

Figure 23B Heart in more detail



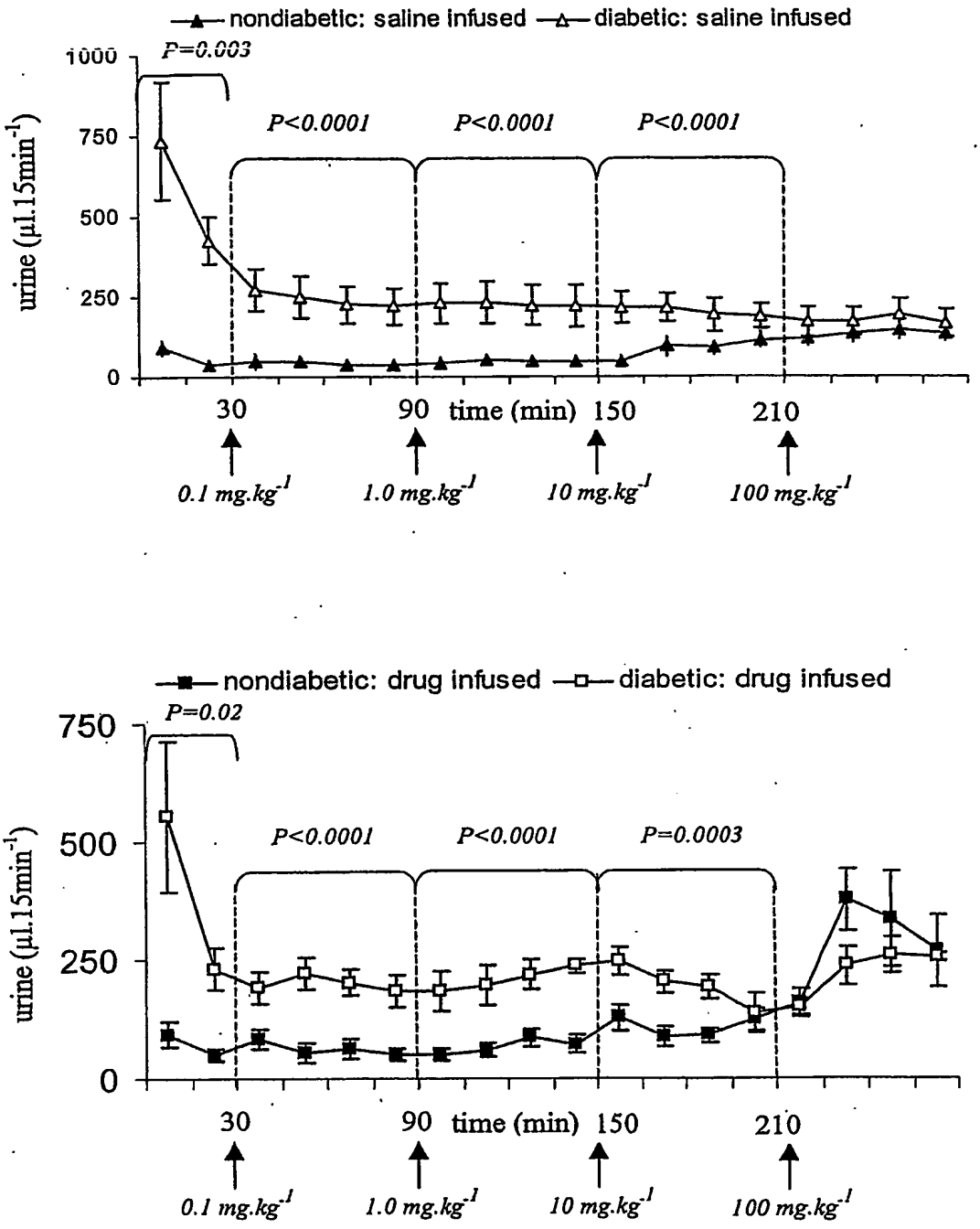


Fig. 24. Urine excretion in diabetic and nondiabetic animals in response to increasing doses of trientine (*bottom;* 0.1, 1.0, 10, 100 mg.kg^{-1} in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline *(top)*. Each point represents a 15 min urine collection period (see Methods for details). Error bars show SEM and *P* values are stated if significant ($P < 0.05$).

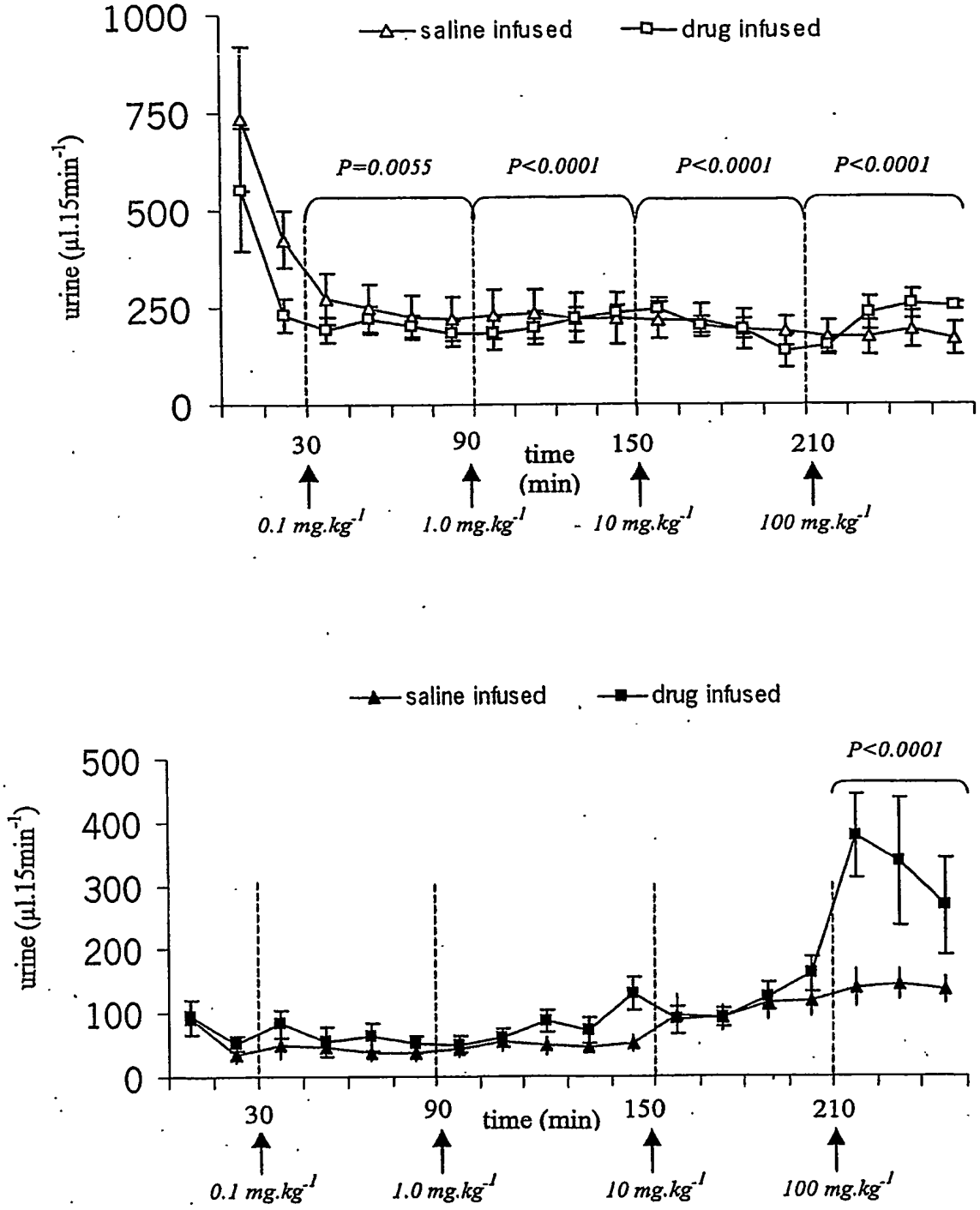


Fig. 25. Urine excretion in diabetic *(top)* and nondiabetic *(bottom)* rats receiving increasing doses of trientine (0.1, 1.0, 10, 100 mg.kg^{-1} in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline. Each point represents a 15 min urine collection period (see Methods for details). Error bars show SEM and *P* values are stated if significant ($P < 0.05$).

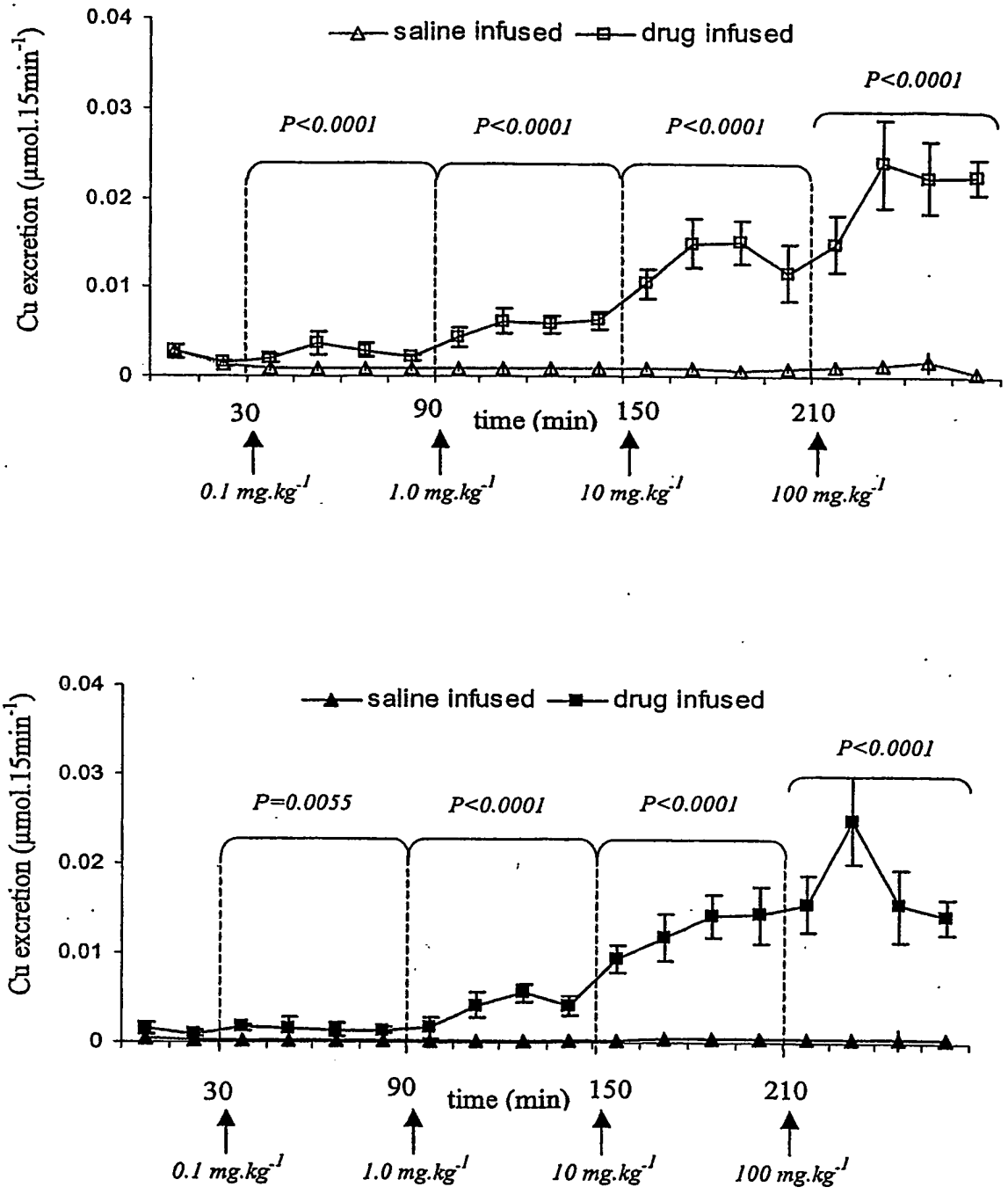


Fig. 26. Copper excretion in urine of diabetic *(top)* and nondiabetic *(bottom)* rats receiving increasing doses of trientine (0.1, 1.0, 10, 100 mg.kg^{-1} in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline. Each point represents a 15 min urine collection period (see Methods for details). Error bars show SEM and *P* values are stated if significant ($P < 0.05$).

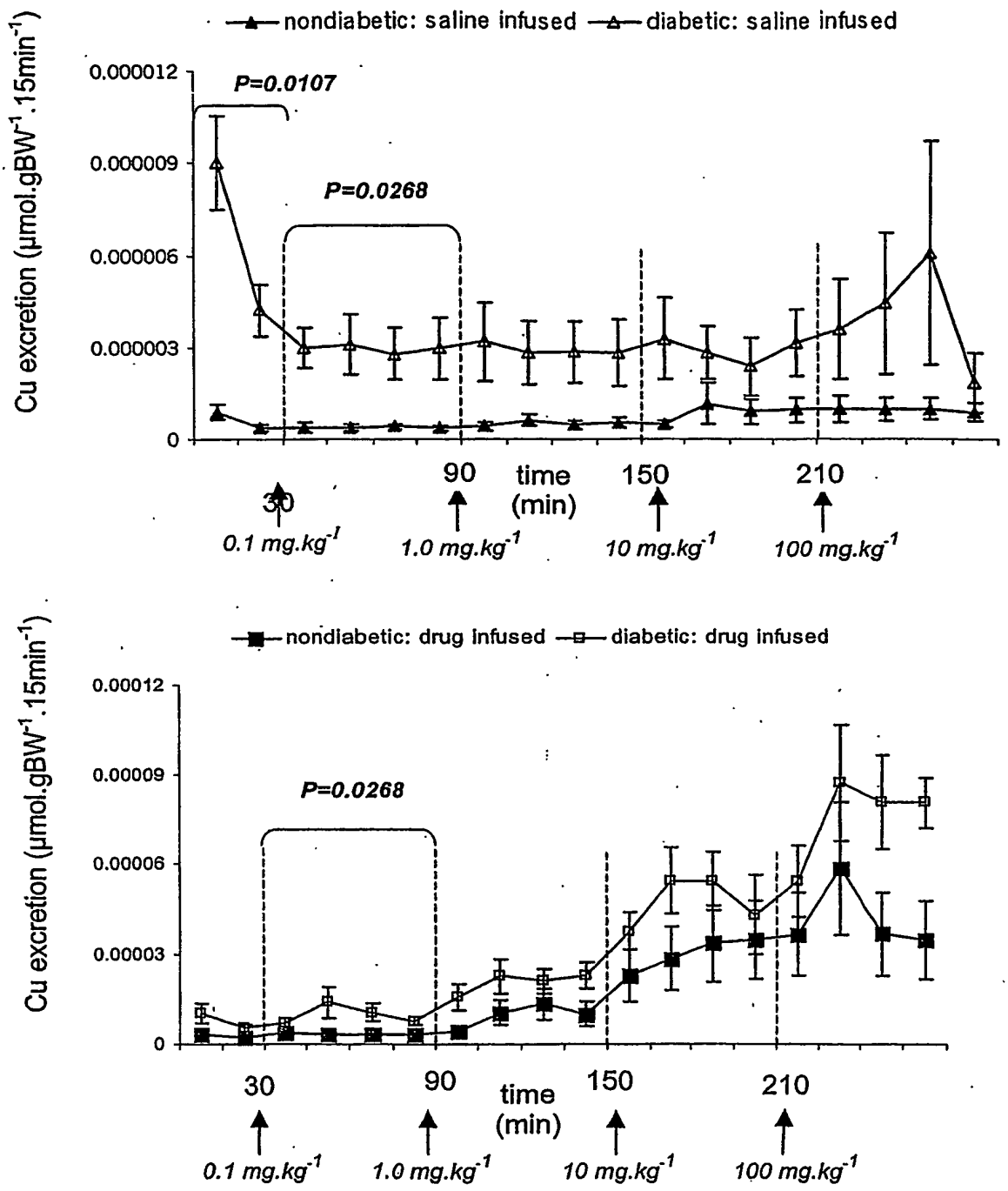


Fig. 27. Urinary copper excretion per gram of bodyweight in diabetic and nondiabetic animals in response to increasing doses of trientine (*bottom;* 0.1, 1.0, 10, 100 mg.kg^{-1} in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline *(top)*. Each point represents a 15 min urine collection period (see Methods for details). Error bars show SEM and *P* values are stated if significant ($P < 0.05$).

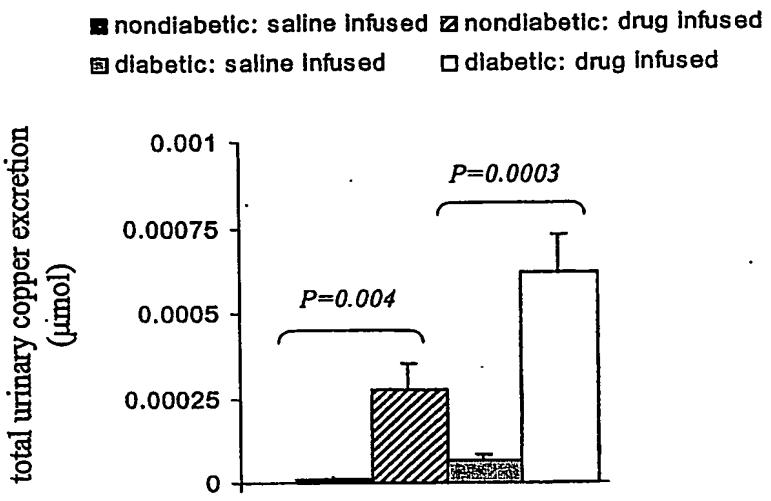


Fig. 28. Total urinary copper excretion (µmol) in nondiabetic animals administered saline (black bar, n = 7) or trientine (hatched bar, n = 7) and in diabetic animals administered saline (grey bar, n = 7) or trientine (white bar, n = 7). Error bars show SEM and *P* values are stated if significant ($P < 0.05$).

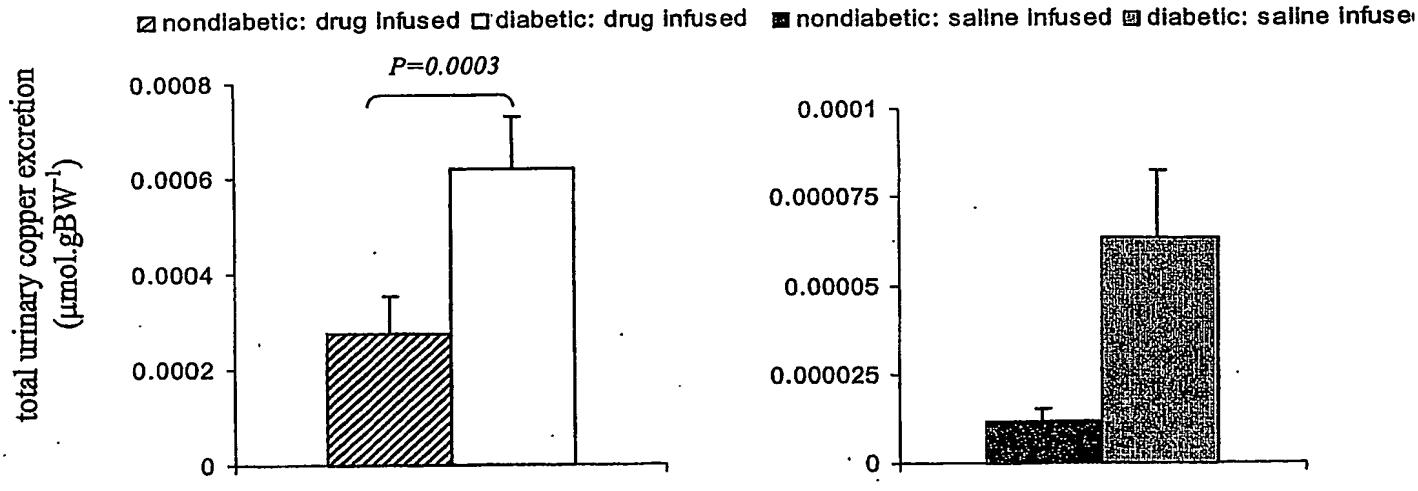


Fig. 29. Total urinary copper excretion per gram of bodyweight ($\mu g.gBW^{-1}$) in animals receiving trientine (nondiabetic: hatched bar, n = 7; diabetic: white bar, n = 7) or saline (nondiabetic: black bar, n = 7; diabetic: grey bar, n = 7). Error bars show SEM and *P* values are stated if significant ($P < 0.05$).

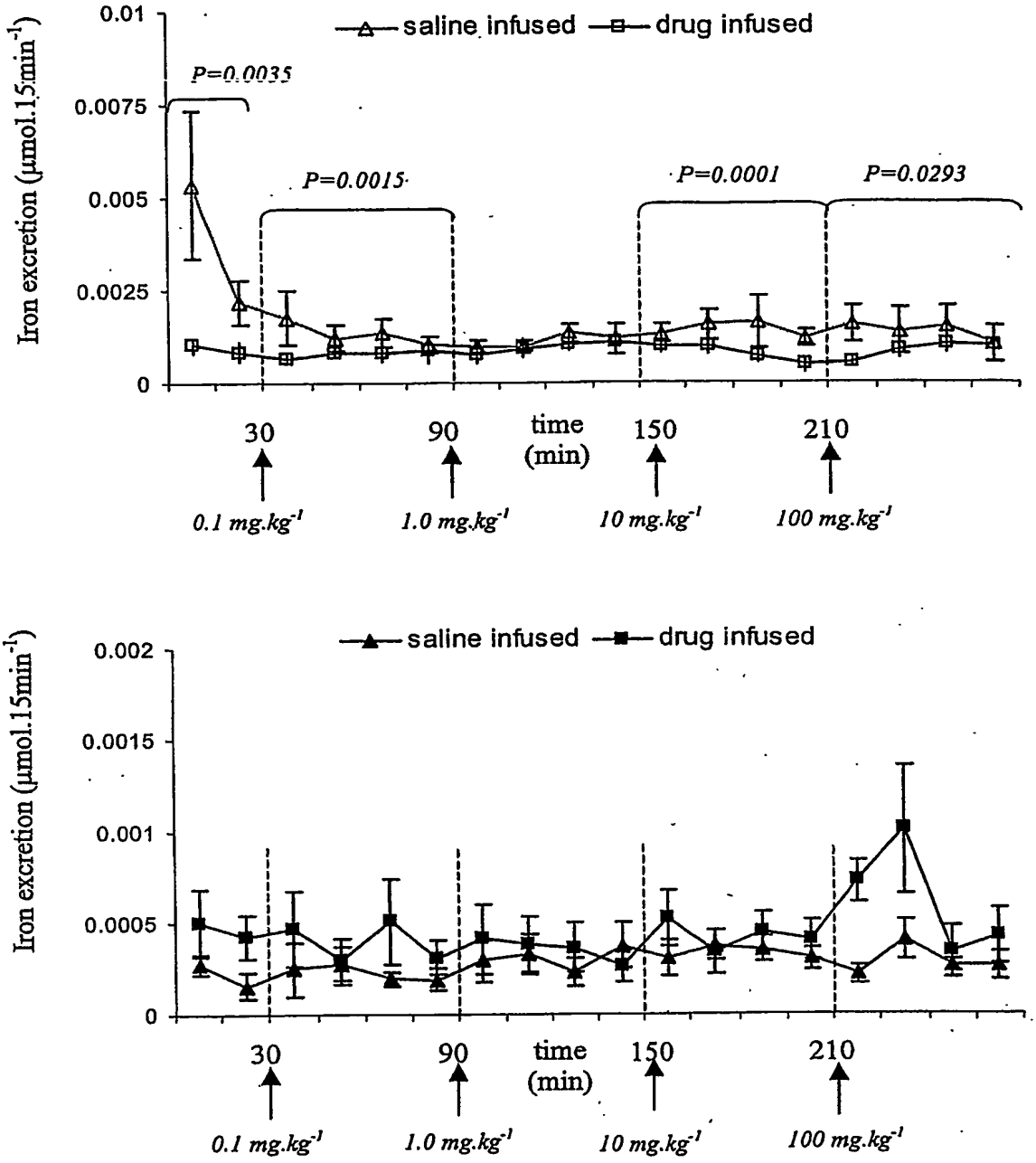


Fig. 30. Iron excretion in urine of diabetic *(top)* and nondiabetic *(bottom)* rats receiving increasing doses of trientine (0.1, 1.0, 10, 100 mg.kg^{-1} in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline. Each point represents a 15 min urine collection period (see Methods for details). Error bars show SEM and *P* values are stated if significant ($P < 0.05$).

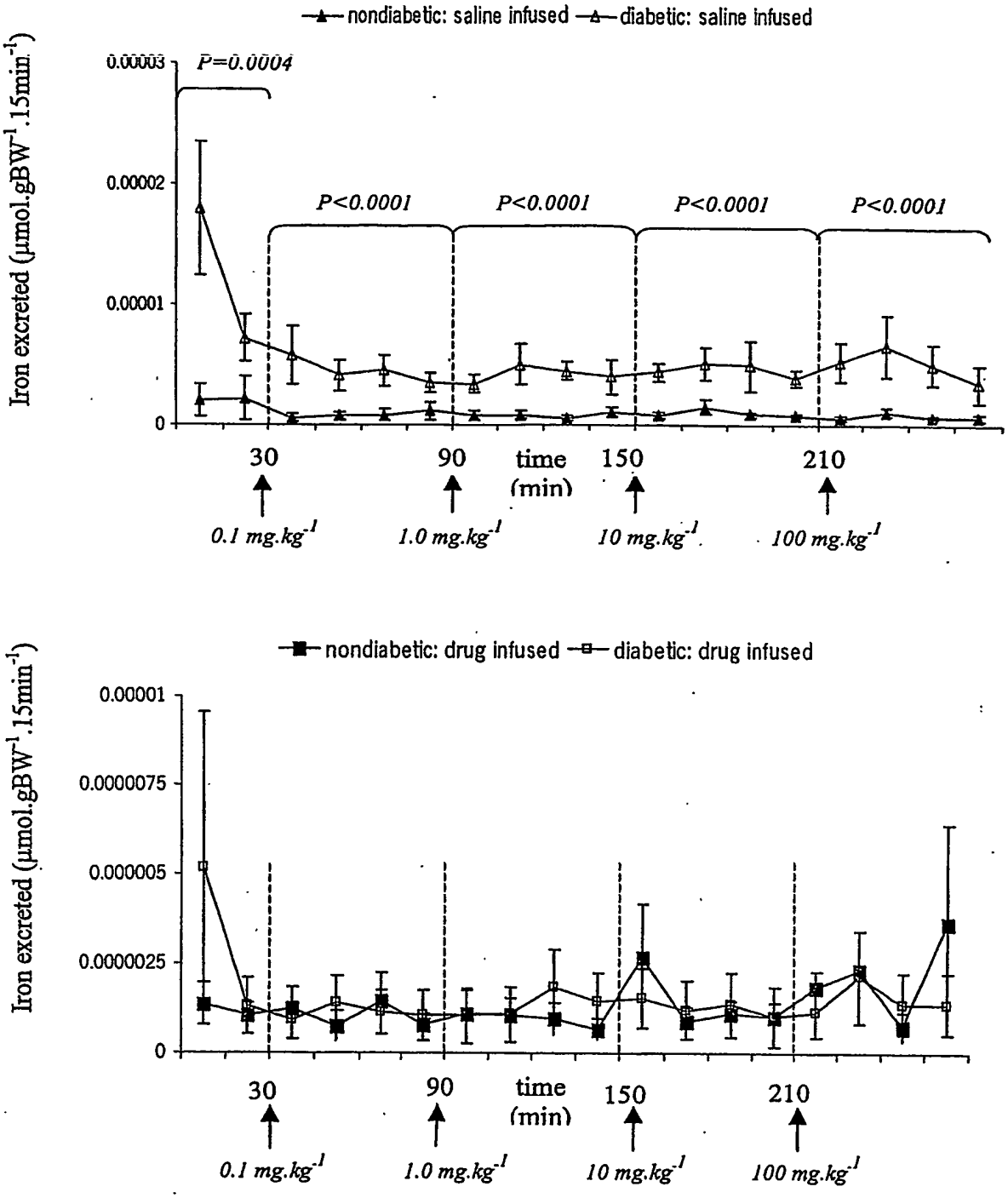


Fig. 31. Urinary iron excretion per gram of bodyweight in diabetic and nondiabetic animals in response to increasing doses of trientine (*bottom;* 0.1, 1.0, 10, 100 mg.kg^{-1} in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline *(top)*. Each point represents a 15 min urine collection period (see Methods for details). Error bars show SEM and *P* values are stated if significant ($P < 0.05$).

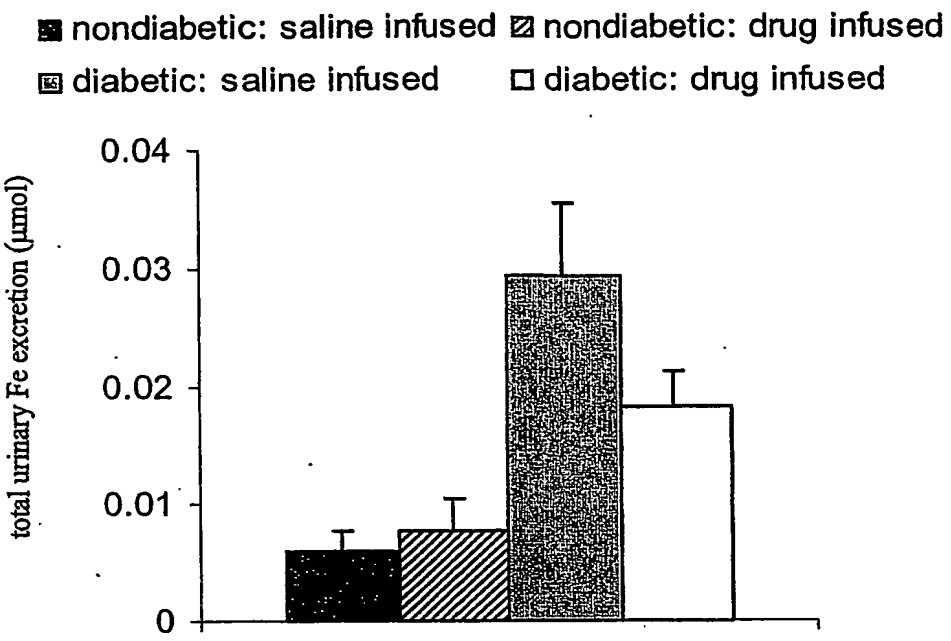


Fig. 32. Total urinary iron excretion (µmol) in nondiabetic animals administered saline (black bar, n = 7) or trientine (hatched bar, n = 7) and in diabetic animals administered saline (grey bar, n = 7) or trientine (white bar, n = 7). Error bars show SEM and *P* values are stated if significant ($P < 0.05$).

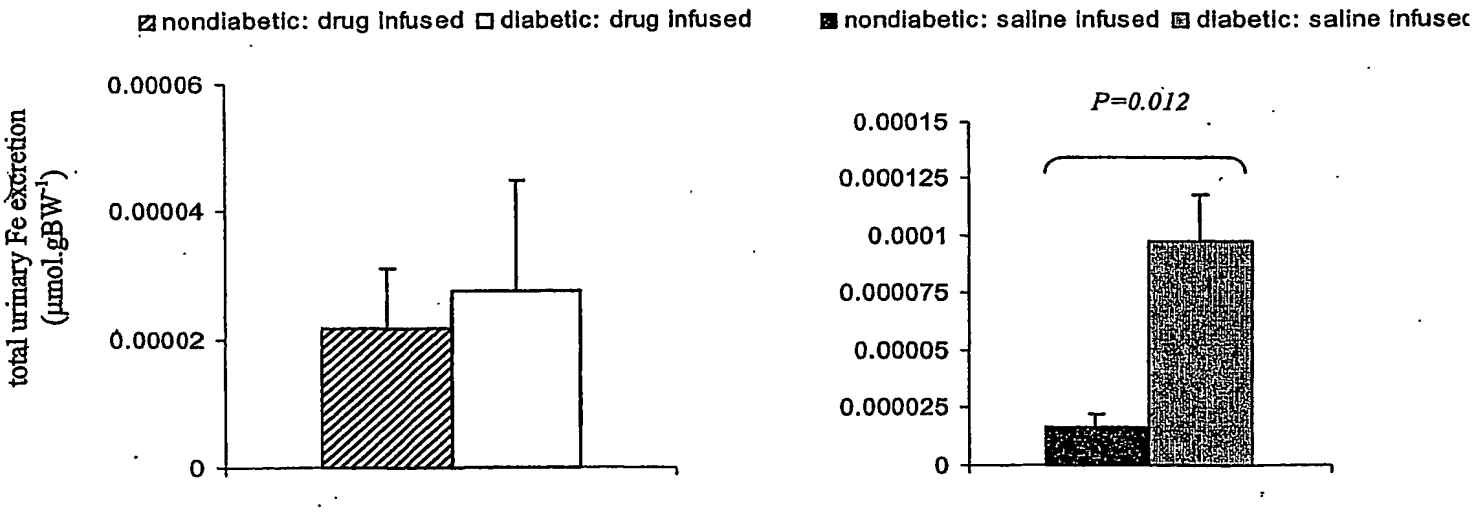


Fig. 33. Total urinary iron excretion per gram of bodyweight ($\mu g.gBW^{-1}$) in animals receiving trientine (nondiabetic: hatched bar, n = 7; diabetic: white bar, n = 7) or saline (nondiabetic: black bar, n = 7; diabetic: grey bar, n = 7). Error bars show SEM and *P* values are stated if significant ($P \leq 0.05$).

Figure 34 Percentage of surviving hearts at each afterload pressure

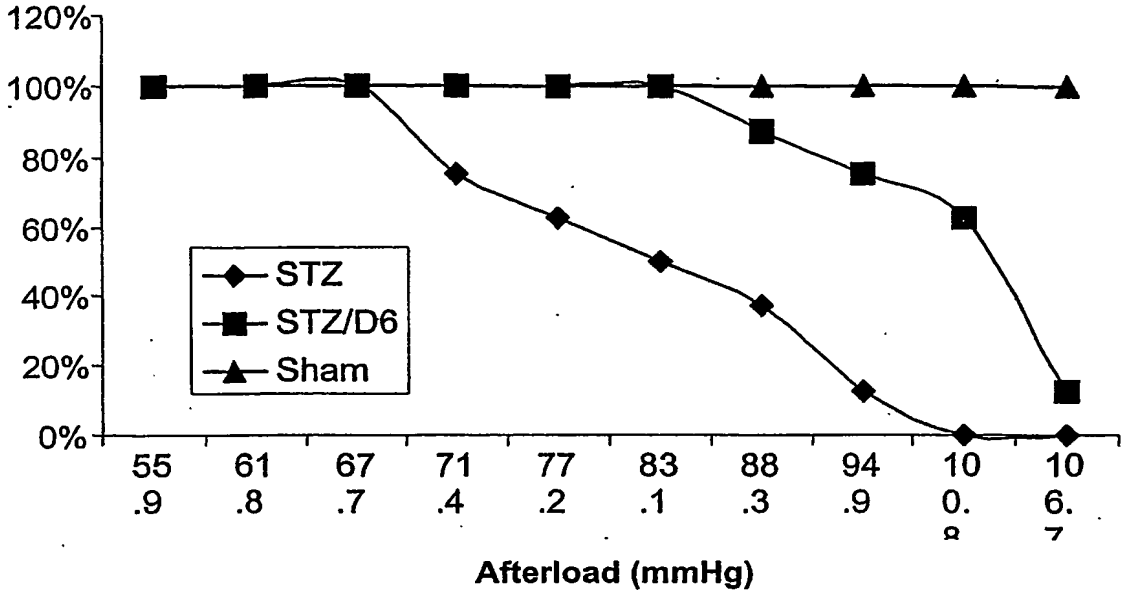


FIGURE 35

Cu excretion			Dose level		
Mixed Model Effects	**Baseline**	**0.1 mg.kg^{-1}**	**1.0 mg.kg^{-1}**	**10 mg.kg^{-1}**	**100 mg.kg^{-1}**
Diabetes	$F_{1,24}$ = 18.52	$F_{1,24}$ = 19.82	$F_{1,24}$ = 21.92	$F_{1,24}$ = 9.93	$F_{1,24}$ = 17.82
(normal/diabetic rats)	P = 0.0002	P = 0.0002	P < 0.0001	P < 0.0001	P < 0.0003
Drug	$F_{1,24}$ = 1.73	$F_{1,24}$ = 24.94	$F_{1,24}$ = 78.36	$F_{1,24}$ = 135.36	$F_{1,24}$ = 162.17
(drug/saline)	*NS*	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
Interaction	$F_{1,24}$ = 0.16	$F_{1,24}$ = 3.58	$F_{1,24}$ = 7.16	$F_{1,24}$ = 6.02	$F_{1,24}$ = 12.43
	NS	*NS*	P < 0.0132	P < 0.0218	P < 0.0017
Sampling time (repeated measure)	t_1, t_2	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4

Fe excretion			Dose level		
Mixed Model Effects	**Baseline**	**0.1 mg.kg^{-1}**	**1.0 mg.kg^{-1}**	**10 mg.kg^{-1}**	**100 mg.kg^{-1}**
Diabetes	$F_{1,23}$ = 12.87	$F_{1,23}$ = 15.82	$F_{1,24}$ = 22.68	$F_{1,24}$ = 14.93	$F_{1,24}$ = 7.35
(normal/diabetic rats)	P = 0.0016	P = 0.0006	P < 0.0001	P = 0.0007	P = 0.0122
Drug	$F_{1,23}$ = 8.6	$F_{1,23}$ = 7.89	$F_{1,24}$ = 12.23	$F_{1,24}$ = 10.91	$F_{1,24}$ = 2.47
(drug/saline)	P = 0.0075	P = 0.01	P < 0.0019	P = 0.003	P = 0.1292
Interaction	$F_{1,23}$ = 12.10	$F_{1,23}$ = 15.06	$F_{1,24}$ = 14.07	$F_{1,24}$ = 17.72	$F_{1,24}$ = 16.76
	P = 0.002	P = 0.0008	P = 0.001	P = 0.0003	P = 0.0004
Sampling time (repeated measure)	2	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4

Plasma concentration-time profiles of trientine after oral administration to four male patients[1]

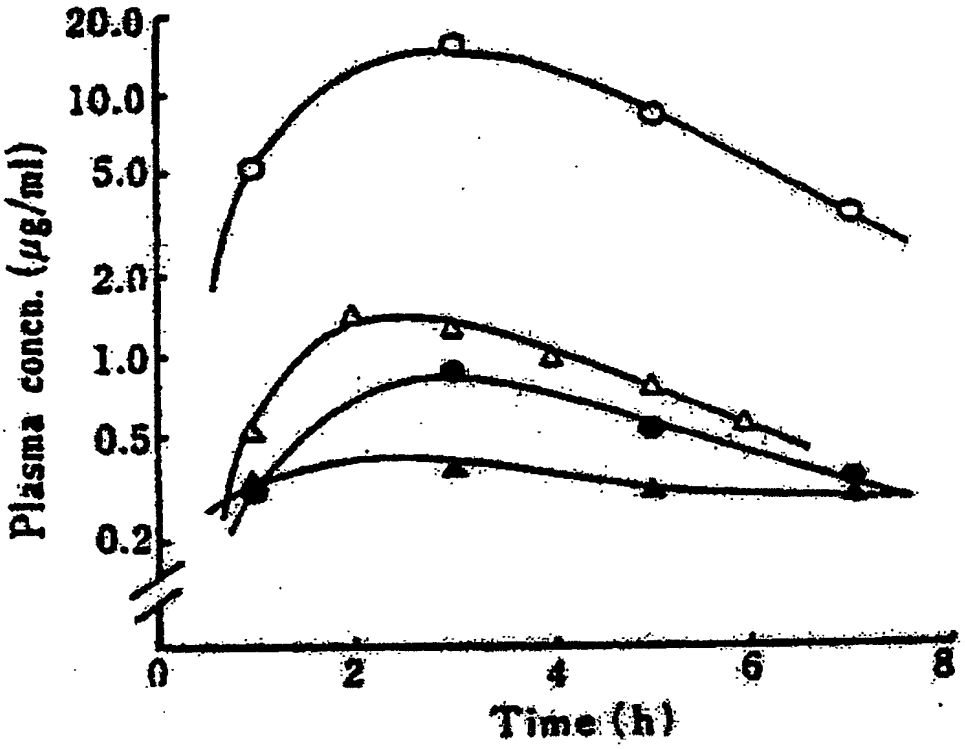


FIGURE 36

Plasma concentration-time profiles of trientine after oral administration to four female patients[1]

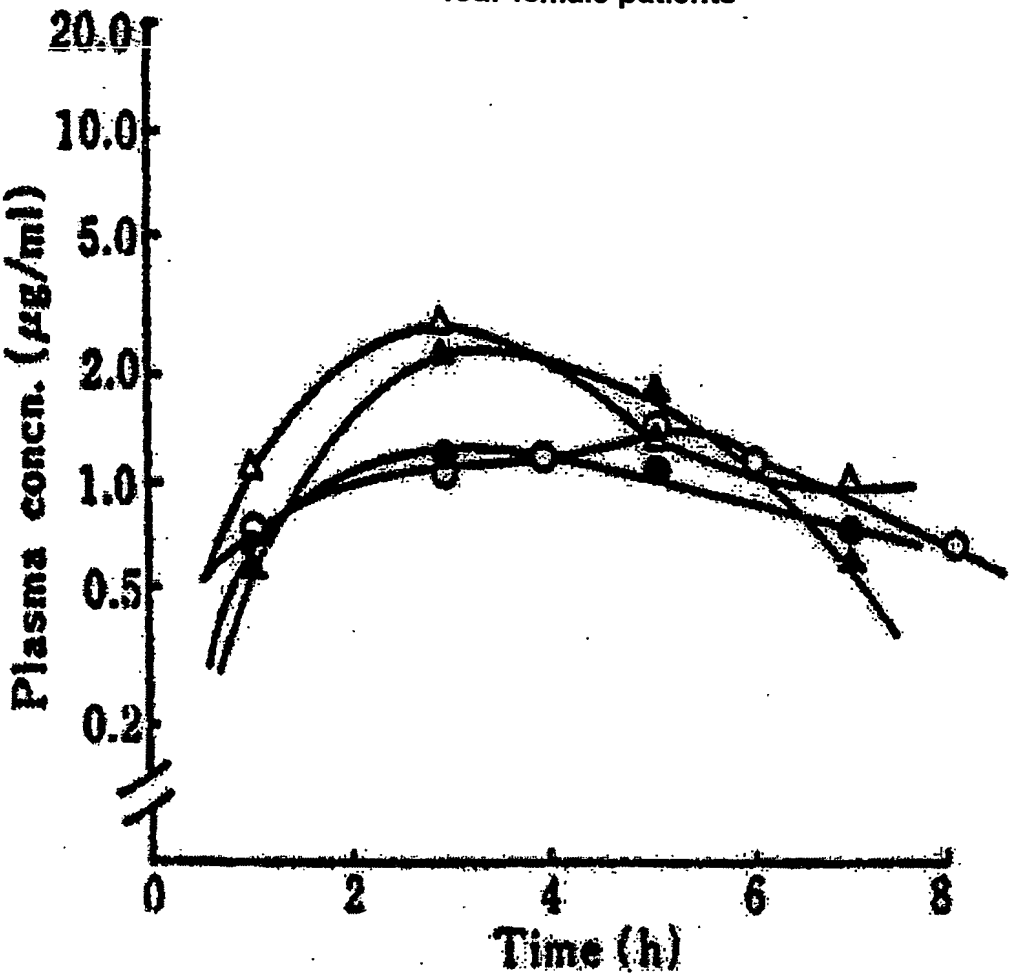


FIGURE 37